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THE ROLE OF THE G PROTEIN-COUPLED RECEPTOR CHEMR23 IN CARDIOVASCULAR INFLAMMATION

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THE ROLE OF THE G PROTEIN-COUPLED RECEPTOR CHEMR23 IN CARDIOVASCULAR INFLAMMATION

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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A mi abuela Pitita y a mi abuelo Toso

ABSTRACT

Cardiovascular inflammation is a biological process characterized by the immune cell response to harmful stimuli within the cardiovascular system, wherein homeostasis is reestablished by neutralizing the insult and promoting the repair of the damaged tissue. However, a failure in the resolution of inflammation can lead to a maladaptive inflammatory response, contributing to the development of cardiovascular diseases such as aortic valve stenosis (AVS) and atherosclerosis, as well as vascular complications such as intimal hyperplasia.

Omega-3 polyunsaturated fatty acids (n-3 PUFA) serve as a substrate for the generation of a group of bioactive specialized pro-resolving lipid mediators (SPMs) that mediate the resolution of inflammation. The SPM resolvin E1 (RvE1) derived from eicosapentaenoic acid reduces cardiovascular inflammation by signaling through the G protein-coupled receptor ChemR23.

The aims of the current thesis were to establish the role of ChemR23 and its activation via RvE1 in the context of AVS, intimal hyperplasia, and atherosclerotic plaque calcification.

In **Article I**, we reported for the first time that n-3 PUFA as well as RvE1 were decreased in calcified regions compared with non-calcified parts of human aortic valves from AVS patients. Stimulation of valve interstitial cells with RvE1 reduced cell calcification after phosphate supplementation. Genetic deletion of ChemR23 in apolipoprotein E-deficient ($Apoe^{-/-}$) mice exacerbated hemodynamic and histological signs of AVS. Moreover, presence of the Fat-1 transgene ($Fat\text{-}1^{tg}$), which enables the endogenous synthesis of n-3 PUFA, halted AVS progression by reducing hemodynamic and histological signs of AVS, and promoting M2 macrophage polarization in the mouse aortic valves. Importantly, $Fat\text{-}1^{tg}$ effects were only detected in the presence of ChemR23, suggesting that the beneficial actions were mediated via RvE1 signaling through ChemR23.

In **Article II,** we showed that ChemR23 genetic deletion in mice increased the development of intimal hyperplasia after a carotid ligation. *In vitro*, ChemR23-deficient vascular smooth muscle cells (VSMCs) exhibited lower proliferation compared with ChemR23 wild-type VSMC. Moreover, ChemR23-deficient macrophages presented a more pro-inflammatory phenotype. Finally, conditioned media transfer from ChemR23-deficient macrophages to VSMCs increased VSMC proliferation compared with conditioned media from ChemR23 wild-type macrophages. This points to a dual effect of ChemR23 in the vasculature depending

on the degree of inflammation, with ChemR23 directly stimulating VSMC proliferation and at the same time suppressing macrophage-induced VSMC proliferation.

In **Article III**, we demonstrated that the presence of Fat-1^{tg} increased n-3 PUFA incorporation in atherosclerotic plaques of Apoe$^{-/-}$ mice, and decreased atherosclerotic plaque calcification. As also seen in aortic valves, ChemR23 deletion increased murine atherosclerotic plaque calcification independently of the local levels of n-3 PUFA, demonstrating the importance of a correct signaling through ChemR23. Moreover, in atherosclerotic plaques of Fat-1^{tg}xApoe$^{-/-}$ mice, the protein expression of the M2-macrophage marker arginase-1 was increased and ChemR23 deletion enhanced the levels of tissue non-specific alkaline phosphatase (TNAP).

In conclusion, the results included in this thesis demonstrate the importance of ChemR23 signaling in cardiovascular inflammation and provide novel mechanistic insights into the beneficial actions of n-3 PUFA and its downstream SPM RvE1 in cardiovascular diseases.

LIST OF SCIENTIFIC PAPERS

I. Artiach G, Carracedo M, Plunde O, Wheelock CE, Thul S, Sjövall P, Franco-Cereceda A, Laguna-Fernandez A, Arnardottir H, Bäck M.
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Circulation. 2020;142:776-789

II. Artiach G*, Carracedo M*, Clària J, Laguna-Fernandez A, Bäck M.
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Front Pharmacol. 2018;9:1327

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Manuscript.

* Both authors contributed equally

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I. Laguna-Fernandez A, Checa A, Carracedo M, Artiach G, Petri MH, Baumgartner R, Forteza MJ, Jiang X, Andonova T, Walker ME, Dalli J, Arnardottir H, Gisterå A, Thul S, Wheelock CE, Paulsson-Berne G, Ketelhuth DFJ, Hansson GK, Bäck M.
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Proteoglycan 4 is Increased in Human Calcified Aortic Valves and Enhances Valvular Interstitial Cell Calcification.
Cells. 2020;9:684

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Upregulated Autophagy in Calcific Aortic Valve Stenosis Confers Protection of Valvular Interstitial Cells.
Int J Mol Sci. 2019;20:1486

* Both authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACE	Angiotensin converting enzyme
Ang-II	Angiotensin II
Apo	Apolipoprotein
$Apoe^{-/-}$	Apolipoprotein E-deficient mouse
Arg1	Arginase 1
α-SMA	Alpha smooth muscle actin
AVA	Aortic valve area
AVS	Aortic valve stenosis
BAV	Bicuspid aortic valve
BMP2	Bone morphogenetic protein 2
BMPR2	Bone morphogenetic protein receptor type 2
CABG	Coronary artery bypass graft
CAC	Coronary artery calcium
CD	Cluster of differentiation
$ChemR23^{-/-}$	ChemR23-deficient mouse
CKD	Chronic kidney disease
CRP	C-reactive protein
CT	Computer tomography
DHA	Docosahexaenoic acid
ECM	Extracellular matrix
EF	Ejection fraction
eNPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1
EPA	Eicosapentaenoic acid
ESC	European Society of Cardiology
EV	Extracellular vesicle
FADS	Fatty acid desaturase
$Fat\text{-}1^{tg}$	Fat-1 transgene
GAG	Glycosaminoglycans

GPCR	G protein-coupled receptor
GWAS	Genome-wide association study
HMOX	Heme oxygenase
ICAM-1	Intracellular cell adhesion molecule 1
IDL	Intermediate density lipoprotein
IF	Immunofluorescence
IHC	Immunohistochemistry
IL	Interleukin
LC-M/SMS	Liquid chromatography tandem mass spectrometry
LDL	Low density lipoprotein
$Ldlr^{-/-}$	Low density lipoprotein receptor-deficient mouse
$Ldlr^{-/-}/ApoB^{100/100}$	Low density lipoprotein receptor-deficient/Apolipoprotein B-100-only mouse
Lp	Lipoprotein
LPC	Lysophosphatidylcholine
LT	Leukotriene
MAPK	Mitogen-activated protein kinases
Mar	Maresin
MCP1	Monocyte chemoattractant protein-1
MGP	Matrix gla protein
MMP	Matrix metalloproteinase
MYH11	Myosin heavy chain 11
n-3 PUFA	Omega-3 polyunsaturated fatty acids
n-6 PUFA	Omega-6 polyunsaturated fatty acids
NFκB	Nuclear factor kappa B
OPG	Osteoprotegerin
OPN	Osteopontin
ORO	Oil red O
oxLDL	Oxidized low density lipoprotein
PALMD	Palmdelphin
PCI	Percutaneous coronary intervention

PCSK9	Proprotein convertase subtilisin/kexin type 9
Pi	Phosphate
PPi	Pyrophosphate
RANK	Receptor activator of nuclear factor-kappaB
RANKL	Receptor activator of nuclear factor-kappaB ligand
Runx2	Runt-related transcription factor 2
RvD1	Resolvin D1
RvD2	Resolvin D2
RvD3	Resolvin D3
RvE1	Resolvin E1
SPM	Specialized pro-resolving lipid mediator
TAVI	Transcatheter aortic valve implantation
TGFβ	Transforming growth factor beta
TNAP	Tissue non-specific alkaline phosphatase
TNFα	Tumor necrosis factor alpha
TOF-SIMS	Time-of-flight secondary ion mass spectrometry
TWAS	Transcriptome-wide association study
VCAM-1	Vascular cell adhesion molecule 1
VEC	Valvular endothelial cell
VIC	Valvular interstitial cell
VSMC	Vascular smooth muscle cell
WT	Wild-type

1 INTRODUCTION

Cardiovascular inflammation is characterized by the biological response of the immune system to harmful stimuli within the cardiovascular system. This process may happen at any level of the cardiovascular tree, for example at the aortic valve or vasculature. Inflammation is therefore a biological defense mechanism in order to reestablish homeostasis. However, a maladaptive inflammatory response may become chronic, thus contributing to the development of inflammatory cardiovascular diseases such as aortic valve stenosis (AVS) and atherosclerosis.

1.1 THE AORTIC VALVE

The aortic valve is an avascular tissue composed by three semilunar leaflets or cusps. The aortic valve leaflets are attached to the aortic wall and open and close with every heartbeat, maintaining the unidirectional blood flow from the left ventricle to the aorta when open during systole, and allowing the optimal refill of the ventricle while preventing regurgitation of blood from the aorta when closed during diastole[1].

The non-coapted region of the valve leaflet is called the belly[2]. Behind the belly of the leaflets, the aortic root gives rise to the sinuses of Valsalva[3], where the coronary arteries begin. During diastole, when the valve is closed, the blood in the aorta exerts a backpressure of 80 mm Hg on the belly of the leaflets, keeping them stretched and completely closed. In systole, the pressure on the leaflets is reduced and they transition into a relaxed state, moving towards the aortic wall[4] (Figure 1). The correct mobility and pliability of the valve, determined by the cellular and molecular micro-architecture, dictates its optimal functionality[5].

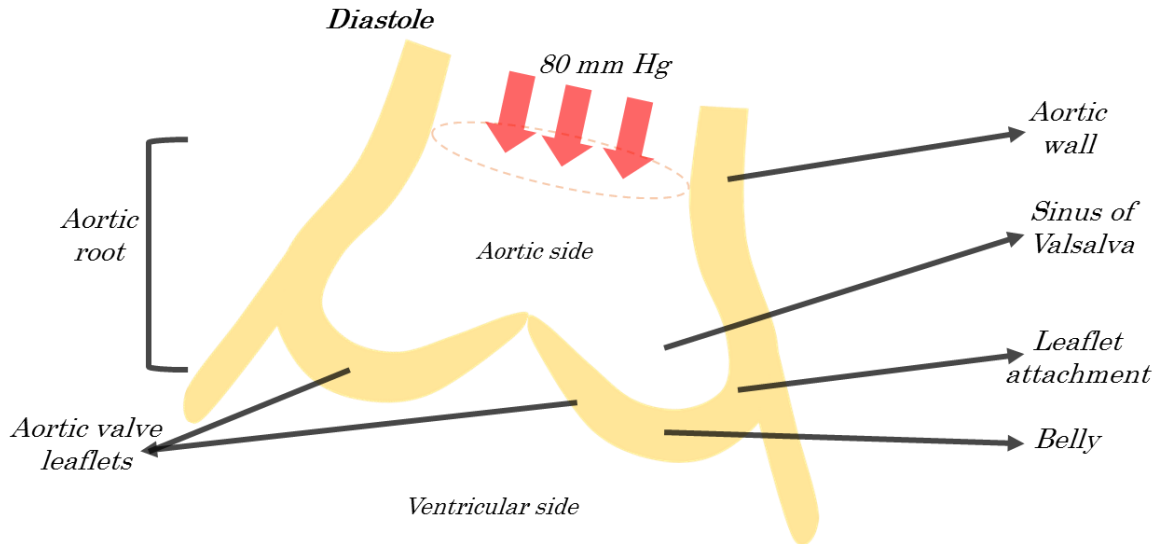

Figure 1. Schematic two dimensional representation of the aortic valve in diastole.

1.1.1 Micro-architecture of the aortic valve

The micro-architecture of the aortic valve leaflets is the intrinsic determinant of a correct valve function and durability[6]. Each leaflet is composed of an external coat layer of valvular endothelial cells (VECs) which covers the surface of both the ventricular and aortic side. On the aortic side, below the endothelium, is the fibrosa layer. The fibrosa is mainly composed of tightly packed collagen fibers and valvular interstitial cells (VICs). Below the fibrosa, a well-organized layer of glycosaminoglycans (GAGs) and VICs exists called the spongiosa. Between the spongiosa and the endothelial layer of the ventricular side is a layer of elastin and VICs called the ventricularis[7] (Figure 2).

Collagen, GAGs and elastin are extracellular matrix (ECM) components with specific and well-defined roles in valve function[8]. Collagen, in particular collagen type I, is important for valve durability[9]. Elastin provides tissue elasticity while GAGs facilitate rearrangements of the other two ECM components in every cardiac cycle, providing cushioning and a shear absorbing function[10].

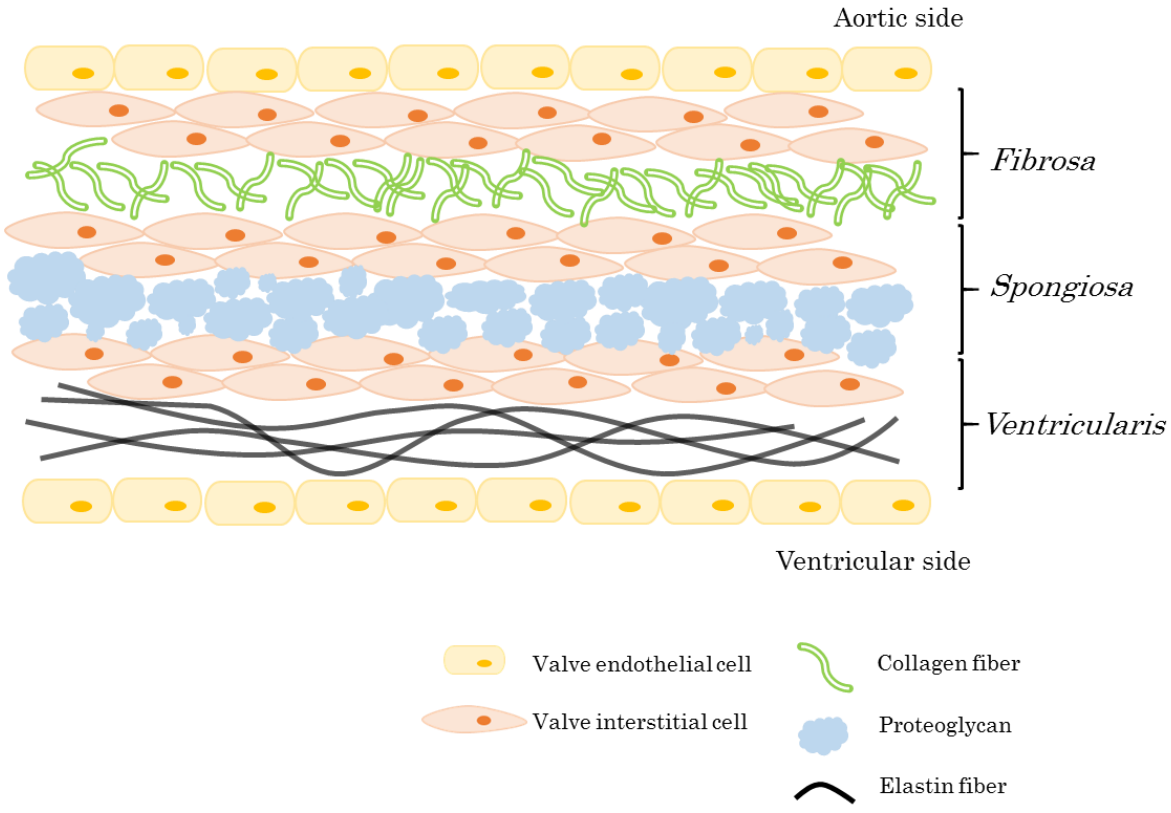

Figure 2. Schematic representation of the different layers of an aortic valve leaflet.

1.1.2 VICs

VICs constitute the main cell population in the aortic valve. This cell type has a mesenchymal origin and possesses the capacity to modify the ECM in order to maintain an optimal aortic valve functionality[11].

At least 5 different types of VICs have been identified within the aortic valve, and are characterized by the presence of different markers: embryonic progenitor endothelial/mesenchymal cells, progenitor VICs (pVICs), quiescent VICs (qVICs), activated VICs (aVICs) and osteoblastic VICs (oVICs)[12].

The embryonic progenitor endothelial/mesenchymal cells have been identified during valve formation during heart development. This process is initiated by the endocardial cushion formation, which is accompanied by an endothelial to mesenchymal transition (EndMT) of endocardial cells during embryogenesis, giving rise to the aortic valve[13, 14].

qVICs are the main population of VICs present in healthy mature human aortic valves. They actively differentiate into the other VIC types under abnormal conditions such as injury or disease[15].

pVICs, or valvular stem cells, are not very well characterized yet, but they may play an important role in aortic valve repair[12, 16].

aVICs express alpha smooth muscle actin (α-SMA), which shows their activation and differentiation into myofibroblast-like cells. aVICs possess the ability to modify ECM components by altering proteolytic pathways through the expression of matrix metalloproteinases (MMPs) and cathepsins, increasing proliferation, and stimulating fibrotic pathways by the expression of cytokines such as transforming growth factor beta (TGFβ)[17].

oVICs are a subset of VICs that actively regulate the calcification process in AVS, a key event in the development of the disease[12], by upregulating the expression of bone morphogenetic protein 2 (BMP2), and runt-related transcription factor 2 (Runx2). This induces the activation of calcification-related signaling pathways such as the Wnt/Lrp5/β-catenin pathway, which in turn increases local calcification[18].

It is important to mention that the classification of VICs into different groups has been primarily defined by the presence immunohistochemical markers. Future studies, using for example single cell sequencing, may provide further insights. For instance, a recent study using single cell-RNA sequencing has shown differential transcriptomic patterns between VICs from

atrioventricular valves and semilunar valves. The same study demonstrates that VICs increase apoptosis during the remodeling phase in the developmental period of the heart[14].

1.1.3 VECs

VECs cover the complete structure of the aortic valve leaflets and promote valve integrity. Despite the similarities between VECs and arterial endothelial cells, they differ in several aspects. For instance, VECs align to blood flow in a perpendicular manner in the response to shear stress, whereas arterial endothelial cells remain parallel[19].

Moreover, the anatomical location of VECs within the aortic valve determines differential transcriptomic profiles. For example, VECs from the ventricular side of the aortic valve contain a higher expression of the atheroprotective gene Kruppel like factor 2 (KLF2) as well as decreased levels of expression of monocyte chemoattractant protein-1 (MCP1)[20]. In contrast, VECs located in the aortic side of the leaflets have a higher expression of proteins involved in calcification and ossification related processes[21].

1.2 AVS

Several registries and epidemiological studies have shown a significant association between age and AVS prevalence, reaching a prevalence of 2 to 7% in individuals above 65 years of age[22] and representing a substantial burden in the elderly population [23-25]. When severe, AVS causes a significant cardiac outflow obstruction with a one-year mortality rate above 50% in severely symptomatic AVS patients[26].

The progressive aortic valve narrowing develops as a consequence of an increased thickening followed by calcification of the aortic valve leaflets[1]. A vast majority of pharmacological clinical trials have failed to stop or regress the disease[27], leaving surgical and transcatheter aortic valve implantation (TAVI) as the only available therapeutic treatment[28].

The risk of AVS increases with age as well as other factors such as male gender[24], hypercholesterolemia[24], hypertension[24], obesity[29] smoking[30], renal dysfunction[31], diabetes, metabolic syndrome[32] and having a bicuspid aortic valve (BAV). BAV is a common congenital abnormality with a prevalence of 1-2% in the population[33]. Moreover, AVS often co-exists with coronary artery disease[34]. However, about 50% of patients with AVS do not have concomitant coronary artery disease[35], indicating that this comorbidity is not completely overlapping. Differential mechanisms between the two cardiovascular diseases are further supported by the lack of effect of for example statins, which are world-leading drugs for

coronary artery disease treatment, but ineffective in slowing down AVS in large clinical trials[36-38].

The morphological hallmark of AVS is, as mentioned previously, an increased thickening of the aortic valve leaflets with concomitant presence of calcified nodules which may extend throughout the whole surface of the aortic valve leaflets (Figure 3).

Figure 3. Calcified aortic valve leaflet – calcification stained with Alizarin red.

Calcification starts at the aortic side of the leaflets, primarily in the attachment points to the aortic wall as a result of being the regions exposed to the highest mechanical stress[1]. The presence of calcified nodules then expand towards the belly of the leaflets, disturbing the organized molecular and cellular cuspal micro-architecture[39]. It is interesting to mention that previous studies have shown different global transcriptional and protein expression signatures when comparing non-calcified and calcified regions of aortic valves from AVS patients[40]. As a consequence of the calcification process, the valve is not capable of the correct opening movement, producing the cardiac outflow obstruction characteristic of the disease. This may be accompanied by an impaired closing movement leading to aortic valve regurgitation.

1.2.1 AVS diagnosis

The evaluation of the aortic valve function for diagnosis of AVS is typically studied in the clinic by echocardiography[41]. This method enables the characterization of the blood hemodynamics in addition to mechanical parameters of the aortic valve, such as aortic valve area (AVA) and transaortic peak velocity, as well as left ventricular functionality[42]. The AVA, defined as the opening orifice area of the leaflets, corresponds to 3-4 cm^2 in a healthy person and is accompanied by a transaortic peak velocity below 1 m/s. The 2017 European Society of

Cardiology (ESC) guidelines define severe AVS by an AVA under 1 cm^2 and a transaortic peak velocity above 4 m/s accompanied by a mean pressure gradient, defined as the difference in blood pressure before and after the aortic valve, which is above 40 mm Hg when left ventricular ejection fraction (EF) is preserved[43]. It must be added that the progression of the disease varies in different patients. For this reason AVS patients are stratified into fast and slow progressors[44]. This is measured by the yearly increase in blood peak velocity, being fast progression when the increase is above or equal 0.3 m/s/year and slow progression when below 0.3m/s/year[43].

1.2.2 Phases of AVS

AVS progression can be divided into two independent phases where several factors such as lipid infiltration, inflammation and physicomechanical forces converge into end-stage calcification of the aortic valve. These two consecutive phases have been described in the literature as the initiation and propagation phase[45-47].

1.2.2.1 Initiation phase

Low shear and high tensile stress give rise to the initiation phase of AVS by inducing a local VEC dysfunction in the aortic side of the aortic valve leaflets[48]. This leads to the infiltration of lipoproteins and apolipoproteins such as apolipoprotein B (ApoB)[49] and lipoprotein (Lp) (a)[50, 51], from the bloodstream to the subendothelial space of the aortic valve[48].

This process is accompanied by a subsequent VEC activation characterized by the overexpression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intracellular cell adhesion molecule 1 (ICAM-1), which leads to inflammatory cell infiltration[52-55]. The fact that these events occur at the aortic side of the aortic valve leaflets may explain why calcified nodules first appear and are more pronounced on the aortic side of the aortic valve leaflets.

Increased oxidative stress is another hallmark of the initiation phase of AVS[56]. The increased oxidative stress in the aortic valve leaflets induces the formation of oxidized low density lipoproteins (oxLDL) and oxidized phospholipids which induce calcification in vascular cells[57-59]. In addition, oxLDL increases the expression of VCAM-1 and ICAM-1, thus further increasing the extravasation of immune cells from the vessel lumen into the aortic valve[49, 53, 60]. Oxidized phospholipids can also be transformed by lipoprotein phospholipase A2 into lysophosphatidylcholine (LPC), which has been associated with increased apoptosis of VICs[61]. This has been further studied in mouse models demonstrating that an increased oxidative stress

induced by angiotensin II (Ang-II) leads to increased calcification in the aortic valve[62]. Interestingly, Ang-II is formed by the action of the angiotensin converting enzyme (ACE), which is upregulated in stenotic valves[63].

Morphological and cellular analyses of stenotic valves have shown an early infiltration of macrophages and other immune cells[39, 64]. Once in the subendothelial space, activated macrophages take up oxLDL and secrete pro-inflammatory cytokines as well as ECM degrading enzymes that may lead to the first macroscopic observations, such as increased aortic valve thickening and early mechanical dysfunction[39]. These observations have received support from studies where increased levels of proteolytic activity prior to the formation of calcification have been identified in early stages of AVS[39].

1.2.2.2 Propagation phase

The impaired ECM formation and degradation, as a consequence of non-resolved local inflammation, can induce the generation of sites for extracellular dystrophic calcification[65], defined as the deposition of calcium and phosphate crystals in soft tissues. Moreover, activated macrophages and VICs actively release calcifying extracellular vesicles (EVs). EVs are enriched with pro-calcifying factors such as tissue non-specific alkaline phosphatase (TNAP) and annexins[66, 67] which facilitate the ion entrance into the vesicle, thus increasing the necessary components for the initiation of mineralization, which ultimately form hydroxyapatite crystals serving as initiation sites for further dystrophic mineralization[68-70].

In the following stages of AVS, the increased inflammation together with the dystrophic calcification further activate VICs and direct them into the myofibroblastic and osteoblastic-like phenotypes[71] through the upregulation of osteogenic pathways, promoting an active initiation of the aortic valve calcification process[72]. Indeed, mouse models with high levels of inflammation, such as apolipoprotein E-deficient ($Apoe^{-/-}$) mice, have lipoprotein infiltration, immune cell infiltration and late-stage calcification in the aortic valve[73].

Myofibroblastic VIC differentiation further activates pathophysiologic pathways which increase the aberrant ECM deposition and degradation. This process occurs primarily at the aortic side of the leaflets (tunica fibrosa) where VICs may have a higher plasticity for differentiating into the cell subtypes mentioned above[40]. VIC myofibroblastic differentiation is characterized by an increased expression of α-smooth muscle actin (α-SMA) and vimentin[74, 75]. VIC myofibroblastic differentiation induced by TGFβ[76] promotes SMAD activation[77, 78], which has been shown to increase GAG chain elongation. GAG chain elongation increases low density lipoprotein (LDL) binding to the ECM[79]. Moreover, during the propagation phase,

activated VICs together with activated macrophages and other immune cells will increase the release of MMPs and cysteine endoproteases, resulting in an aberrant disruption of the ECM fibers (collagen and elastin) which serve as a scaffold and substrate for dystrophic calcification nodules.

In the last stages of AVS, oVICs are responsible for the heterotopic bone formation characteristic of the late stages of the calcification process of the aortic valve. oVICs are characterized by having an increased expression of the osteoblastic markers Runx2[80] and BMP2[18, 72]. BMP2 plays an important role in the initiation of the osteoblastic differentiation whereas Runx2 is a master regulator of skeletal development and osteogenesis. BMP2 belongs to the TGFβ superfamily, which, through its receptor bone morphogenetic protein receptor type 2 (BMPR2) induces the phosphorylation and activation of SMAD1/5/8, proteins that will further activate genes involved in the differentiation into osteoblastic-like cells such as Runx2, Osteopontin (OPN) and TNAP[81], enzyme that promotes the conversion of the calcification inhibitor pyrophosphate (PPi) to free phosphate (Pi)[82]. Runx2 expression has been found in cells surrounding calcified particles in macroscopically healthy valves, linking its expression to an early osteoblastic differentiation of valvular cells[83]. Moreover, the role of Runx2 in osteoblastic differentiation has been shown in animal models, where calcification was inhibited after its genetic deletion[84], demonstrating the importance of Runx2 in the calcification process.

1.2.3 Genetic studies in AVS

Several genetic studies have been performed in order to identify genes which play determinant roles in AVS. The lipoprotein (A) (LPA) locus, which encodes for Lp (a) was identified in a genome-wide association study (GWAS) to be associated with incident AVS and prevalent aortic valve calcium[85]. Furthermore, a transcriptome-wide association study (TWAS) identified a palmdelphin (PALMD) variant to be associated with AVS[86]. Finally, a variant in the fatty acid desaturase (FADS)1/2 locus has been associated with AVS and valvular calcification[87], with accompanying changes in transcription and fatty acids locally in the aortic valve[88].

1.2.4 AVS treatment

As mentioned before, there is no current pharmacological treatment to cure nor halt AVS. Traditionally, surgical aortic valve replacement has been the method used for these patients. However, during the last decade, TAVI has emerged as a less-invasive method for valve replacement. TAVI was initially considered for patients who were not suitable for traditional

valve replacement due to co-morbidities[89], but is now expanding its indication to moderate and low risk patients.

Randomized clinical trials testing the role of statins in patients with AVS have shown that reduction of LDL cholesterol does not alter echocardiographic parameters nor calcification measured by computer tomography (CT)[36-38], despite the evidence that LDL cholesterol is associated with disease progression[24]. However, Lp (a) was found to be increased in patients treated with rosuvastatin[90], suggesting that this increase may have masked any positive effect of the reduced LDL cholesterol.

Due to the lack of effect of statins and their possible effect in increasing Lp (a), proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors have emerged as potential and promising new candidates as a pharmacological treatment of AVS[91, 92]. Indeed, by increasing the levels of low density lipoprotein receptor (LDLr), PCSK9 inhibitors achieve a reduction in LDL cholesterol levels by more than 50%. Interestingly, and in contrast to the observations in statins, PCSK9 inhibitors also reduce the levels of Lp (a)[93, 94].

For this reason, a deeper understanding of the biological processes and molecular mechanisms underlying AVS is needed for the future development of novel pharmacological therapies.

1.3 THE ARTERIAL WALL

The arterial wall is organized in three different layers: the tunica intima, media and adventitia[95]. The tunica intima is the innermost layer and is in direct contact with the blood flow. Formed by a thin monolayer of vascular endothelial cells, this layer is responsible for immune cell recruitment by the surface protein expression of adhesion molecules. The tunica media is mainly composed by elastin fibers and vascular smooth muscle cells (VSMCs).

VSMCs are responsible for maintaining the contractile tone of the arteries, and can be divided into two groups based on their phenotype. The contractile phenotype is characterized by a high presence of α-SMA and myosin heavy chain 11 (MYH11), as well as a limited proliferation rate. On the other hand, the synthetic phenotype is characterized by less contractile ability and increased proliferation and migration, as wells as increased MMP secretion and collagen production[96]. Under certain conditions, other VSMC subtypes have been identified, for example the osteoblastic phenotype, which in similarity to oVICs are characterized by upregulating pro-calcification genes. This phenotype is associated with vascular calcification[97, 98].

The tunica adventitia, being the outermost layer, is mainly composed of connective tissue, adipose cells and fibroblasts. This layer is responsible for providing physical support to the vasculature (Figure 4).

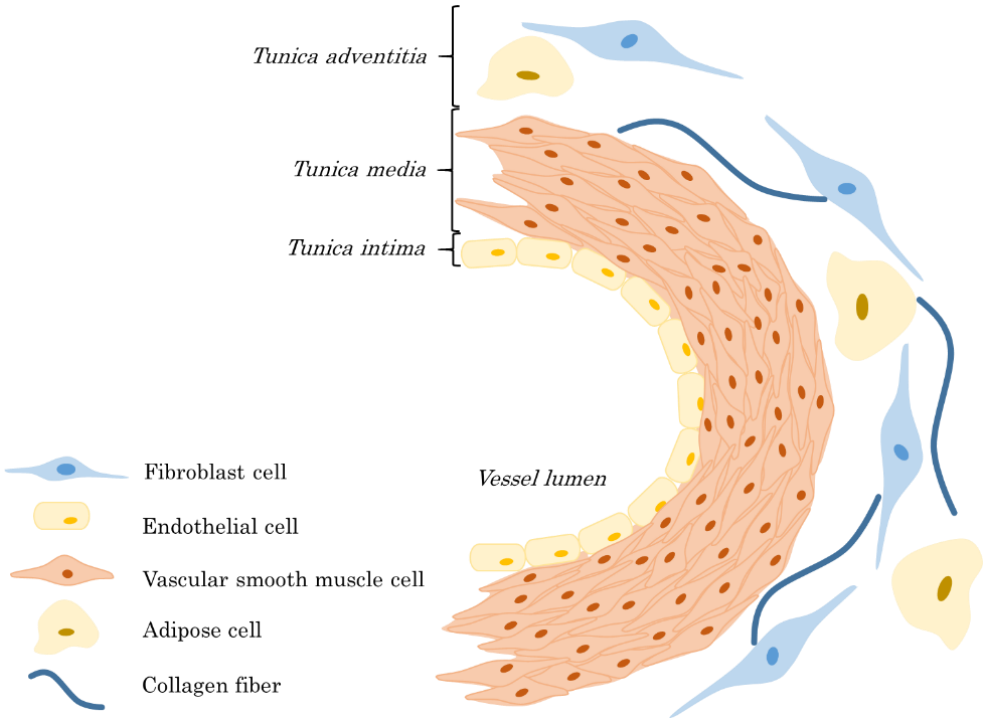

Figure 4. Schematic representation of the different layers of the arterial wall.

1.3.1 Vascular injury and intimal hyperplasia

Intimal hyperplasia is a reparative process within the arterial wall as a response to a hemodynamic or mechanical injury. The primary pathology of intimal hyperplasia is characterized by the migration of VSMCs from the media layer into the subendothelial space of the artery, accompanied by an increased VSMC proliferation[99], which ultimately leads to stenosis and occlusion of the vessel (Figure 5).

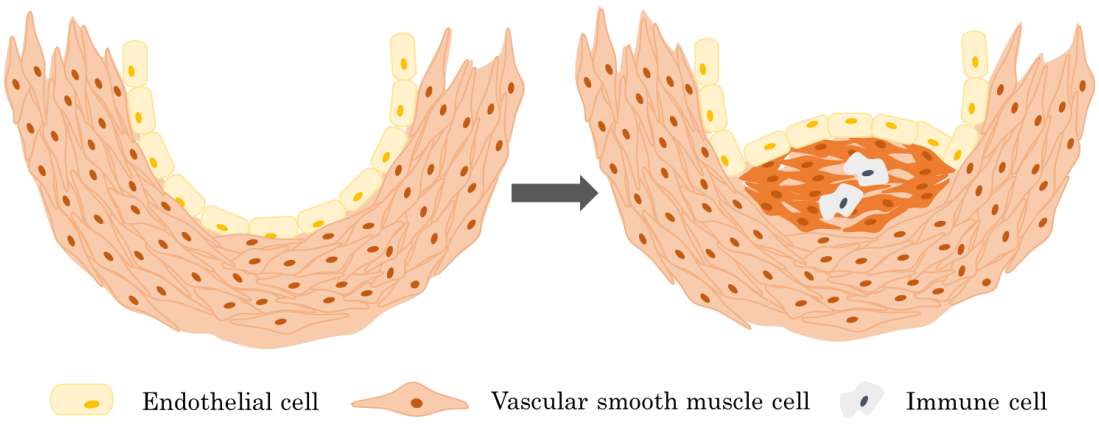

Figure 5. Schematic representation of intimal hyperplasia in the arterial wall.

After vascular injury, for example a coronary artery bypass graft (CABG) failure or in stent placement after coronary intervention, leukocytes attach to the endothelium and infiltrate the arterial wall. This leads to the secretion of pro-inflammatory molecules and ECM degrading enzymes, as well as growth factors that further increase the response by locally recruiting more leukocytes to the site of injury[100] and modulating VSMC behavior into the synthetic phenotype, thus inducing activation, migration and proliferation.

The newly formed intima layer, also named neointima, is characterized by the presence of VSMCs positive for α-SMA, and fibroblasts, accompanied by an aberrant ECM deposition[101, 102]. Moreover, immunohistochemical evaluation of intimal hyperplasia injury sites has identified an increased presence of neutrophils, lymphocytes and macrophages[103], as well as cytokines and MMP secretion[104]. Despite the pronounced thickening of the arterial wall by the newly formed intima layer, the tunica media remains thin with no apparent changes in morphology nor cell composition[105].

Currently, treatment for intimal hyperplasia relies on the effectiveness of drug eluting stents for the prevention of restenosis after percutaneous coronary interventions. Stents are coated with cell cycle inhibitors such as rapamycin and paclitaxel, therefore blocking VSMC proliferation. However, intimal hyperplasia still remains a clinical problem in surgical procedures, for example in CABG failure[106].

1.4 ATHEROSCLEROSIS

Atherosclerosis is the underlying cause of coronary artery disease[107] whereas cerebrovascular atherosclerosis is one of the causes of stroke. In similarity to AVS, atherosclerosis is an inflammatory disease characterized by the early infiltration and deposition of cholesterol-containing LDL within the vascular wall followed by a pronounced infiltration of inflammatory cells[108].

The initiation of the atherosclerotic plaque starts with the infiltration of cholesterol-containing LDL in the vascular wall through the intima[109]. The LDL particles located in the subendothelial space are subjected to oxidation by the action of myeloperoxidase, lipoxygenase, and reactive oxygen species[110]. The accumulation of oxLDL particles in the subendothelial space leads to the subsequent activation of the endothelial cells, whereby the expression of adhesion molecules such as VCAM-1 is increased[111]. As a response, circulating monocytes are recruited to these sites. Once in the subendothelial space, monocytes differentiate into macrophages and start phagocyting oxLDL particles[112]. The unending engulfment of oxidized particles leads macrophages to produce more inflammatory cytokines which results in an increased

recruitment of more macrophages and other inflammatory cells. Activated macrophages differentiate into foam cells which stay trapped in the subendothelial space, leading to the characteristic chronic inflammation process of this disease[113] that drives the formation and maturation of the atherosclerotic plaque.

Moreover, dendritic cells take up antigens in the plaque and migrate to secondary lymphoid organs in the vicinity where they present the antigens to naïve T-cells through MHC class II antigen presentation. T-cells differentiate into several subtypes, which play different roles in the development of the atherosclerotic plaque. Furthermore, the presence of anti-oxLDL antibodies has been detected in atherosclerotic plaques, demonstrating the role of B-cells in mediating responses against atherosclerosis plaque[114]. Indeed, vaccination targeting oxLDL exerts a beneficial effect against atherosclerosis in preclinical animal models[115, 116].

The presence of dead cells forming necrotic areas is characteristic of advanced atherosclerotic lesions[117]. The accumulation of dead cells is the result of the combination of both increased apoptosis and a reduced efferocytosis, the latter meaning clearance of the apoptotic cells[118]. Advanced atherosclerotic plaques are also characterized by increased calcification, which is as an independent predictor of cardiovascular mortality[66, 119, 120], specifically plaques with increased microcalcifications. Indeed, atherosclerotic plaque microcalcifications have been proposed to play a critical role in plaque stability and to be a risk factor for plaque rupture compared with large and well-established calcifications[121, 122].

The formation of calcified nodules within the atherosclerotic plaque resembles those observed in aortic valves described above, by means of impaired ECM deposition, generation of sites for dystrophic mineralization, EVs released by macrophages and VSMC which ultimately form hydroxyapatite crystals[123], and VSMC differentiation into osteoblastic-like cells.

A stable fibrous cap consisting of VSMCs and collagen covers the atherosclerotic plaque keeping it isolated from the bloodstream and protected from rupture. Though the triggers are not fully understood, local breakdown of the collagen fibers by the action of MMPs together with VSMC death and increased calcification promote the weakening and subsequent rupture of the fibrous cap[124], or alternatively atherosclerotic plaque erosion which is defined as the absence of endothelium leading to acute thrombus formation without signs of plaque rupture[125]. In the case of plaque rupture, the inner content of the atherosclerotic plaque is released into the vessel lumen, which interacts with the bloodstream, inducing local platelet aggregation and a subsequent thrombus formation[124, 126]. The thrombus may lead to coronary artery occlusion, causing myocardial infarction, or stroke if this happens in a cerebrovascular artery.

1.4.1 Atherosclerosis treatment

Currently, pharmacological intervention for atherosclerosis management relies on lipid management by statins[127]. Despite the clear effect of statins in reducing inflammation[128], evidence suggests that their beneficial effects rest primarily in their potential to reduce lipids rather than in targeting inflammation.

Targeting inflammation as an option for reducing cardiovascular risk has been widely studied, and was recently highlighted by a clinical trial that demonstrated that directly targeting inflammation may serve as a potential treatment for cardiovascular risk reduction[129]. The study involved patients with a history of myocardial infarction, and the results showed that the risk of recurrent cardiovascular events was lower among those who received anti-inflammatory therapy by the interleukin (IL) 1β monoclonal antibody (Canakinumab) compared to patients that received placebo. However, one of the adverse effects of this therapy was that patients were more prone to infections due to neutropenia and had significantly more deaths due to infection or sepsis.

Another recent clinical trial has also shown that patients with chronic coronary disease receiving a low-dose of colchicine, an anti-inflammatory drug, had a significantly reduced cardiovascular risk compared with placebo treated patients[130], supporting the beneficial effects of targeting inflammation in atherosclerosis. However, this approach is not yet implemented in clinical therapy for patient management. Moreover, recent evidence has shown that high levels of the omega-3 polyunsaturated fatty acid (n-3 PUFA) eicosapentaenoic acid (EPA) significantly decreases ischemic events, including cardiovascular death[131]. This observation will be discussed in more detail in the coming chapters of this thesis.

In advanced coronary atherosclerotic disease and in acute coronary syndromes, either CABG or percutaneous coronary intervention (PCI) with stent implantation is used when the atherosclerotic plaque causes a significant vessel obstruction or after plaque rupture.

1.5 MOLECULAR MECHANISMS OF CARDIOVASCULAR CALCIFICATION

Several molecular mechanisms take place during the mineralization process in cardiovascular diseases. Most of these molecular mechanisms are common to all types of cardiovascular calcification. For this reason, the following section applies to both aortic valve calcification and atherosclerotic plaque calcification.

During embryo bone development, secreted Wnt binds to the LDL co-receptor Lpr5[132]. This activates β-catenin that translocates to the cell nucleus, activating bone-formation related genes

expression. Experimental studies have identified a high expression of Wnt in human and murine atherosclerotic plaques[133], as well as elevated levels of Lpr5, β-catenin and Wnt3a ligand in valves of AVS patients compared with non-calcified valves, suggesting a reactivation of the Wnt/β-catenin pathway[134].

Notch1, 2, 3 and 4 are transmembrane receptors that play a crucial role in the regulation of ossification processes. Notch pathways suppress Wnt/β-catenin signaling. Specifically, the intracellular domain of Notch1 binds directly to β-catenin, suppressing the expression of Runx2 and as a consequence the ossification process[135]. Indeed, mutations in Notch1 have been described to be associated with AVS[136], and Notch1 overexpression reduces osteoblastogenesis by suppressing Wnt signaling[135].

Under homeostasis, the pro-calcification molecular pathways in the vasculature are repressed by the action of calcification inhibitors. Within this family, some of the most important inhibitors are Matrix gla protein (MGP), Klotho, Osteoprotegerin (OPG) and PPi.

MGP is produced locally by structural cells and its function depends of the carboxylation of the protein glutamate residues by vitamin K. Indeed, patients treated with vitamin K antagonists develop higher coronary and valvular calcification[137, 138]. Furthermore, MPG has been shown to be decreased in human calcified valves from AVS patients compared with non-calcified valves[139]. This has been further observed in murine models where deletion of MGP induces cardiovascular calcification. When MGP is carboxylated, it actively inhibits calcification by directly binding to BMP or to free calcium ions[140]. Mechanistically, *in vivo* studies in rats have shown that warfarin treatment promotes cardiovascular calcification by inhibiting the recycling of vitamin-K, thus suppressing the subsequent carboxylation of MGP[141]. As a matter of fact, warfarin administration in rats is a commonly used model for cardiovascular calcification[142, 143].

Klotho is a transmembrane protein which acts as a co-receptor for fibroblast growth factor 23 (FGF23)[144]. FGF23 signaling through the heterodimer Klotho and FGF receptor decreases phosphate resorption via down-regulation of the protein levels of the renal proximal tubule type-II sodium phosphate co-transporters as well as $1,25(OH)_2$ vitamin D synthesis. Moreover, Klotho can be also present in soluble form in circulation. The functions of soluble Klotho have been associated with Wnt signaling inhibition[145], as well as with promoting endothelial integrity in the vasculature[146].

Furthermore, OPG plays a role in inhibiting cardiovascular calcification by acting as a decoy receptor for the receptor activator of nuclear factor-kappa B (NFκB) ligand (RANKL)[147]. In

the absence of OPG, RANKL binds to the transmembrane receptor RANK and intracellularly signals the expression of osteogenic gene circuits.

PPi is derived from the hydrolysis of ATP by the action of the ectonucleotide pyrophosphatase/phosphodiesterase 1 (eNPP1). PPi inhibits mineralization by substituting phosphate (Pi) in the calcified nodule[148]. Loss of function of eNPP1 has been associated with generalized arterial calcification in infants[149]. Moreover, preclinical studies have shown that eNPP1 deletion reduces atherosclerotic plaque calcification[150]. On the other hand, in extracellular fluids, TNAP is the enzyme in charge of limiting the availability of PPi by hydrolyzing it into Pi[151], promoting the deposition and growth of phosphate crystals[152] (Figure 6). TNAP activity is tightly controlled by macrophages, which under certain conditions mediate anti-calcifying actions by downregulating its expression[153]. Furthermore, TNAP overexpression increases medial calcification in ex vivo vascular models[154]. Specific endothelial overexpression of TNAP has also been shown to increase vascular calcification[155].

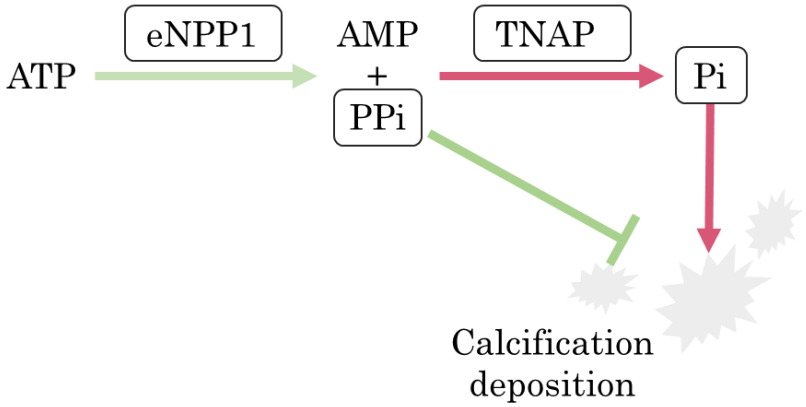

Figure 6. Schematic representation of the opposing functions of eNPP1 and TNAP in the process of calcification deposition.

1.6 CALCIFICATION DETECTION

Several methodologies can be used in order to study the presence of calcification both in atherosclerotic plaques as well as in aortic valves from patients with AVS.

As mentioned before, echocardiography is the first-line evaluation method for assessing the severity of AVS. However, resting echocardiographic assessment of AVS patients may be discordant, especially in the elderly population[156], giving rise to medical uncertainties[157]. For this reason, the interest in calcification assessment of the aortic valves of those patients has increased in the last years.

Computed tomography (CT) is a widely used method for the study of calcification in cardiovascular diseases. Indeed, CT calcium scoring of the coronary arteries has become a well-established method for coronary artery calcium (CAC) volume content and density evaluation in atherosclerotic plaques, which currently serves as a powerful predictor of adverse clinical events[158, 159]. Aside from being used in patients with atherosclerosis, CT has also emerged as a method for the study of aortic valve calcium in patients with AVS. Several studies have confirmed the linear correlation between CT results and calcium content from explanted valves, suggesting this method is a powerful tool for aortic valve calcium quantification in AVS patients[160, 161].

For this reason, CT in aortic valves has emerged as a potential complementary tool to echocardiography for AVS severity assessment due to its independence of hemodynamic patient status. Using CT in AVS patients is part of the recommendations in the latest ESC guidelines for the management of valvular heart disease[43]. However, it ought to be mentioned that one of the limitations of CT in AVS patients is that it ignores the presence of non-calcific leaflet thickening, mostly a consequence of pro-fibrotic events, which also significantly contributes to the pathology and progression of AVS, especially in women [162].

CAC scoring by CT provides information on atherosclerotic plaques for risk prediction of future cardiovascular events. Despite the fact that CAC volume measurement of atherosclerotic plaques provides a direct association with cardiovascular events[163], CAC density inversely correlates with the same outcome, suggesting that a low CAC density (characteristic of microcalcifications) is a potential risk factor for cardiovascular events resulting from atherosclerotic plaque rupture[164].

1.7 N-3 PUFA AND THE RESOLUTION OF INFLAMMATION

The infiltration of lipids, lipoproteins and inflammatory cells are hallmarks of the chronic inflammatory process in cardiovascular diseases. Importantly, cardiovascular inflammation is characterized by a failure in the resolution of the inflammation process. The resolution of inflammation is an active, well-orchestrated process that actively stops and resolves the inflammatory response, decreasing leucocyte recruitment and promoting efferocytosis[165, 166]. Resolution of inflammation is driven mainly by macrophages and other immune cells. However, structural cells also play an important function throughout the process.

When an insult occurs, the inflammation/resolution response is divided into two phases. The first phase is the initiation of the inflammation, characterized by the infiltration of immune cells into the damaged or infected area, followed by an increased production of pro-inflammatory

molecules such as the pro-inflammatory lipid mediators leukotrienes (LTs) and prostaglandins derived from the n-6 PUFA arachidonic acid (AA) [64]. In the second phase, the resolution of inflammation phase takes over to halt the inflammatory process and bring the system to homeostasis in an active manner. The resolution of inflammation is mediated by several molecules such as IL-10, annexin A1[167], nitric oxide, hydrogen sulfide[168], carbon monoxide[169], and with particular importance for this thesis, by specialized pro-resolving lipid mediators (SPMs)[170]. Derived from long chain PUFA, mostly n-3 PUFA, SPMs signal through specific cellular G protein-coupled receptors (GPCRs) [171]. If these signaling circuits fail, inflammation will not resolve and damage will persist and chronify[165].

By the action of the cyclooxygenase and lipoxygenase metabolism, n-3 PUFA-derived SPMs are enzymatically metabolized into the SPM-resolvin, maresin and protectin families which have the potential of initiating the resolution phase, importantly without inducing immunosupression[171].

Specifically, n-3 PUFA serve as a substrate for the generation of the docosahexaenoic acid (DHA)-derived D- series resolvins (RvD), maresins (Mar) and protectins as well as the EPA-derived E-series resolvins (RvE)[102] (Figure 7).

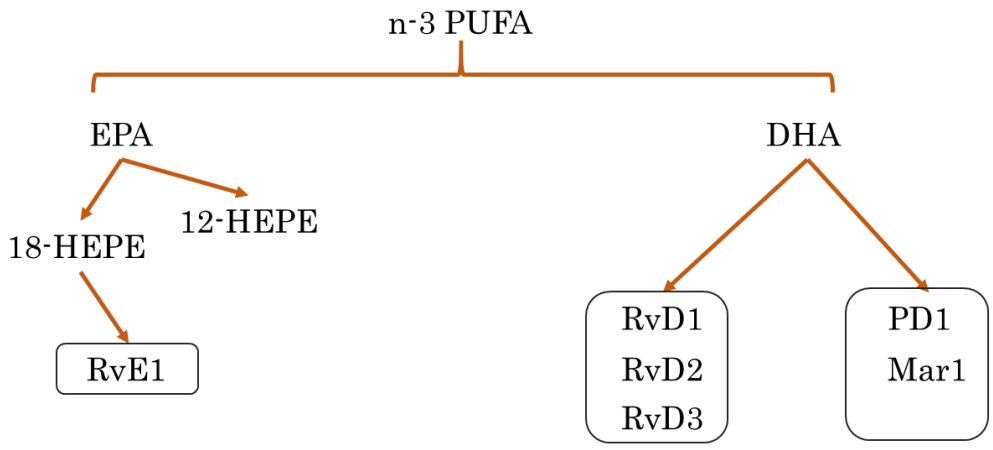

Figure 7. SPMs derived from the n-3 PUFA EPA and DHA.

A fine balance between the pro-inflammatory lipid mediators derived from n-6 PUFA and the SPMs derived from n-3 PUFA may be decisive for disease outcome. For example, the LTB_4/RvD1 ratio is higher in vulnerable regions of human atherosclerotic plaques, being those more prone to rupture[172].

As mentioned before, the implication of n-3 PUFA as a possible treatment for cardiovascular inflammation has been explored during the last years. However, n-3 PUFA treatment for cardiovascular risk reduction has been controversial. Indeed, despite a clinical trial showing

that 1 g/day dietary supplementation of n-3 PUFA led to a clinically important significant benefit in reducing cardiovascular risk[173], other clinical trials testing low concentrations of n-3 PUFA (≤1 g/day) have not revealed protective effects when compared with placebo[174-176]. However, another clinical trial using a higher dose of pure EPA (1.8 g/day) combined with statin therapy in patients with hypercholesterolemia revealed that the risk of ischemic events was significantly lower in the EPA treated group compared with placebo[177]. Furthermore, the beneficial effects of higher doses of pure EPA (4 g/day) in cardiovascular disease prevention have been recently explored in another large randomized controlled clinical trial, demonstrating that EPA treatment in patients already receiving statin therapy conferred a 25% relative risk reduction in major cardiovascular events compared with placebo[131]. Interestingly, another recent study has shown that EPA treatment (4 g/day) slowed atherosclerotic plaque progression and induced plaque regression[178]. These results therefore point to a dominant role of EPA, and not DHA, in reducing cardiovascular risk.

Moreover, other studies have shown that higher levels of C-reactive protein (CRP) in coronary artery disease are associated with a lower intake of n-3 PUFA as well as a lower n-3 PUFA/n-6 PUFA ratio[179]. This observation has also been replicated in healthy volunteers with a high n-6 PUFA/n-3 PUFA ratio intake, who have been shown to have increased CRP levels and elevated inflammatory markers[180]. These observations may reflect a potential imbalance between n-3 PUFA-derived SPMs and n-6 PUFA-derived pro-inflammatory lipid mediators[181] (Figure 8).

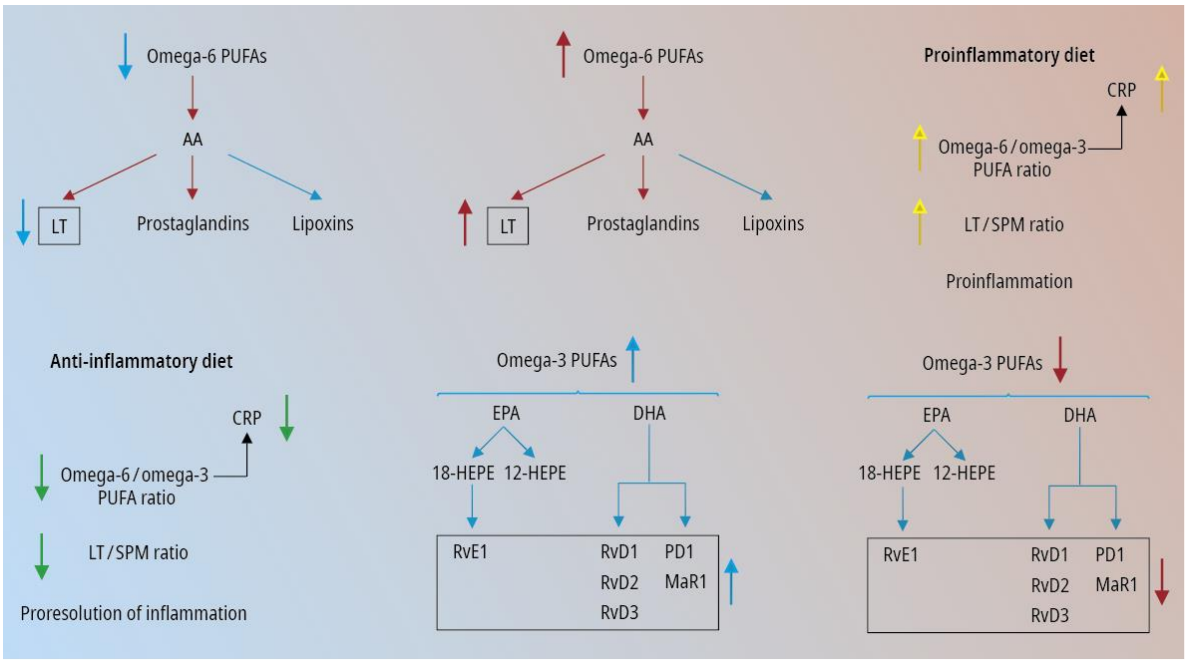

Figure 8. Potential impact of dietary intake of n-3 and n-6 PUFA. Blue color stands for predominant pro-resolution of inflammation. Red color stands for predominant pro-inflammation. Reproduced from Artiach et al., *Kardiol Pol*. (2020)[181].

1.7.1 RvE1 and ChemR23 in cardiovascular inflammation

SPMs exert their actions through specific GPCRs. Specifically, ChemR23 and BLT1 serve as receptors for RvE1; FPR2/ALX and GPR32 for RvD1 and RvD3; and GPR18 for RvD2[165, 182] (Figure 9).

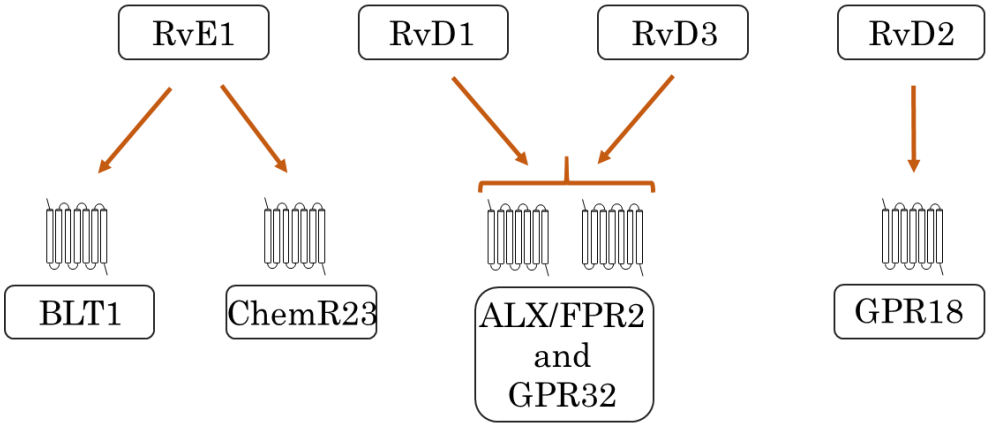

Figure 9. Specific GPCRs for RvE1 and for the D-series RvD1, RvD2 and RvD3.

ChemR23 is a GPCR coupled to $G_{i/o}$, which leads to inhibition of adenylyl cyclase followed by cAMP accumulation, intracellular calcium release, and subsequent phosphorylation of mitogen-activated protein kinases (MAPK) [183, 184]. ChemR23 has been identified in several cell types including macrophages[185], dendritic cells[186], natural killer cells[187] as well as chondrocytes[188], osteoblasts[189], endothelial cells[190] and VSMCs[191]. Although ChemR23 was originally described as one of the receptors for chemerin[183], subsequent studies identified ChemR23 as also having a similar affinity for RvE1[192].

Chemerin is a chemoattractant protein for M1 macrophages[193] mainly found in adipose tissue and associated with several diseases such as chronic kidney disease (CKD) and diabetes. Chemerin levels have also been shown to be increased in coronary artery disease patients not taking low dose aspirin[194]. Moreover, increased chemerin levels increase blood pressure in mice[195]. However, other studies have shown that chemerin exerts opposite biological functions. For example, chemerin levels in CKD patients have been associated with lower coronary calcium[196], as well as with survival in patients receiving dialysis[197]. Interestingly, chemerin-derived peptides resulting from its cleavage have been reported to induce similar functions as RvE1[184].

RvE1 promotes M2 macrophage polarization characterized by the increased expression of arginase 1 (Arg1), Cluster of differentiation (CD) 206, CD163 and CD36 and their anti-inflammatory actions[198]. Recent studies from our group and others have described that RvE1 protects against atherosclerosis development and VSMC calcification[102].

In atherosclerosis, RvE1 modifies macrophage behavior through ChemR23[199] as well as reduces atherosclerotic plaque development[200]. In addition, the genetic deletion of ChemR23 increases atherosclerotic plaque size and inflammation in Apoe$^{-/-}$ mice[199].

In the context of intimal hyperplasia, RvE1 treatment actively promotes M2 macrophage polarization, reducing neointima formation after arterial injury in animal models[198, 201]. Moreover, Pi-induced VSMCs calcification is reduced after RvE1 treatment via ChemR23 signaling[202], pointing to the RvE1/ChemR23 axis as a key disrupted pathway in the development of cardiovascular diseases.

1.7.2 RvE1 and ChemR23 in cardiovascular calcification

As mentioned previously, macrophages are key drivers of the process of cardiovascular calcification. Indeed, extracellular Pi supplementation induces, as a counter response, the generation of a specific subset of macrophages with anti-calcifying properties, characterized by a decreased gene expression and protein activity of TNAP, and accompanied by a sharp increase in gene expression of the M2 marker Arg1 and of the anti-calcifying ectoenzyme eNPP1. Interestingly, the latter changes induced by Pi supplementation were accompanied by a sharp induction of ChemR23 expression (6.1-fold) [153, 203], suggesting that ChemR23 could play an important role in anti-calcifying macrophages. This is explored in more detail later in the present thesis.

SPMs derived from n-3 PUFA may have an important translational implication from a therapeutic point of view for cardiovascular calcification. Previous human studies have shown that higher n-3 PUFA serum levels are associated with a lower incidence of coronary artery calcification[204]. In line with this, experimental *in vivo* studies have shown that EPA administration in rats prevents macrophage infiltration as well as warfarin-induced vascular calcification by suppressing MMP9 activity[205]. Experimental studies from other laboratories have shown that supplementation with EPA also suppresses Wnt signaling, decreasing BMP4 and AXIN2, while upregulating Klotho mRNA levels in mice[206]. Moreover, Klotho$^{-/-}$ mice upregulate the endogenous levels of RvE1 after EPA supplementation and decrease calcium volume scores in thoracic and in abdominal aortas assessed by micro CT[207].

Little is known about EPA and its downstream molecule RvE1 in the development of inflammation-dependent cardiovascular calcification. For this reason, understanding the role of RvE1 as well as ChemR23 in the interplay between structural and inflammatory cells in cardiovascular calcification is therefore needed. This may open up new opportunities for the development of novel therapies for inflammation-dependent cardiovascular diseases in which calcification plays a major detrimental role, such as AVS and atherosclerosis.

2 AIMS

The studies included in this thesis aimed to unravel the role of ChemR23 in cardiovascular inflammation.

The specific aims were:

I. To elucidate the role of the n-3 PUFA/RvE1/ChemR23 signaling axis in the development of AVS (**Article I**).

II. To determine the role of n-3 PUFA and ChemR23 in the development of intimal hyperplasia (**Article II**).

III. To establish the role of n-3 PUFA and the derived SPM RvE1 signaling through ChemR23 in atherosclerotic plaque calcification (**Article III**).

3 EXPERIMENTAL METHODOLOGY

3.1 AVS – HUMAN AORTIC VALVES

Tricuspid aortic valves from AVS patients undergoing aortic valve replacement at Karolinska University Hospital (Solna, Sweden) were collected. The study presented in the current thesis using this material was approved by the local ethics committee (2012/1633) and was in agreement with the Declaration of Helsinki. All patients gave informed consent.

Macroscopic evaluation of the aortic valves allowed us to visually identify and further dissect non-calcified and calcified regions. This approach allowed us to establish a model of the disease continuum within each individual, going from the healthy or fibrotic initial stage (non-calcified tissue) to the advance stage characterized by the presence of bone nodules.

In the experiments presented in current thesis, valve tissue was used for gas chromatography, liquid chromatography tandem mass spectrometry (LC-MS/MS), or for transcriptomic analysis using Gene Chip Affymetrix human transcriptome 2.0. Aortic valve leaflets were also used for immunohistochemical analysis.

3.2 AVS – *IN VIVO* MODELS

In order to understand the biological and molecular mechanisms that drive human AVS, animal models of the disease are needed. Some large animals, such as swine, can develop AVS similarly to humans[208]. However, conducting research in large species is time-consuming and costly, thus leaving them as inefficient models for the study of the disease. For this reason, mouse models are currently the most widely used *in vivo* models for AVS due to their low cost and easy management as well as the existence of a wide variety of genetically modified strains.

Ideally, AVS in mouse models should resemble the pathological hallmarks observed in humans to a certain degree. For example increased leaflet thickness, presence of calcification and inflammatory cell infiltration such as macrophages, together with endothelial activation as well as impaired ECM deposition. Moreover, AVS in mice should also mimic the hemodynamic characteristics of human AVS by means of increased transaortic valve peak velocity as well as decreased cusp separation or distance in systole.

3.2.1 Hypercholesterolemic mouse models

Different AVS models have been developed and used throughout the last decades for AVS research. Aged hypercholesterolemic mice, which are also used to model human

atherosclerosis, are widely used for AVS modeling since these mice mirror the age-associated AVS progression observed in humans[209, 210].

Apoe$^{-/-}$ mice have been described to develop AVS at advanced ages both with and without supplementation with a high-fat diet[39, 73]. However, Apoe$^{-/-}$ mice develop AVS faster when fed a high fat diet compared with mice fed chow diet. Similar to the human disease, this model develops an impaired aortic valve opening, observed by an increased transaortic peak velocity, associated with age. These mice also develop an increased valve thickening with concomitant presence of calcification, as well as immune cell infiltration in terms of macrophages[211] together with endothelial activation observed by increased VCAM-1[39, 73]. Furthermore, Apoe$^{-/-}$ mice express early changes related with AVS progression and prior to calcification, for example, increased levels of calcification-related proteins such as osteocalcin, Runx2 and TNAP as well as proteolytic activity[212].

Low density lipoprotein receptor-deficient/Apolipoprotein B-100-only (Ldlr$^{-/-}$/ApoB$^{100/100}$) mice also develop AVS in late stages of their lives[58, 62]. Ldlr$^{-/-}$/ApoB$^{100/100}$ mice, as well as Apoe$^{-/-}$ mice, develop lipid deposition in the aortic valve leaflets, accompanied by monocyte infiltration and calcification. Interestingly, Ldlr$^{-/-}$/ApoB$^{100/100}$ mice develop AVS in younger stages of life compared with Apoe$^{-/-}$ mice even without being fed a high-fat diet.

Moreover, Ldlr$^{-/-}$ mice fed a high fat diet develop increased valve thickening and early markers of calcification. However, there is no current evidence of hemodynamically significant stenosis in these mice[213-215].

3.2.2 Non-hypercholesterolemic mouse models

Notch1$^{-/+}$ mice fed a high fat diet have been used to study aortic valve calcification processes. However, they do not develop AVS hemodynamically[216]. Interestingly, these mice are reported to be born with a BAV associated with concomitant aortic valve malformations.

MGP$^{-/-}$ and Klotho$^{-/-}$ mice have been widely studied in the context of vascular calcification. Klotho$^{-/-}$ mice develop aortic valve calcification[217, 218]. There is no current evidence demonstrating the presence of aortic valve calcification in MGP$^{-/-}$ mice[219].

In 2014 a new model of AVS was described induced by a mechanical injury to the aortic valve using a spring wire. The valve injury was performed by inserting the wire through the right carotid artery into the left ventricle, followed by moving the wire manually in and out 20 times and rotating it 50 times while monitored by echocardiography in B-mode (Figure 10). Interestingly, these mice started developing hemodynamical changes 1 week after injury, as well as increased thickening, immune cell infiltration, calcification and hemodynamically significant changes after 16 weeks of injury[220, 221].

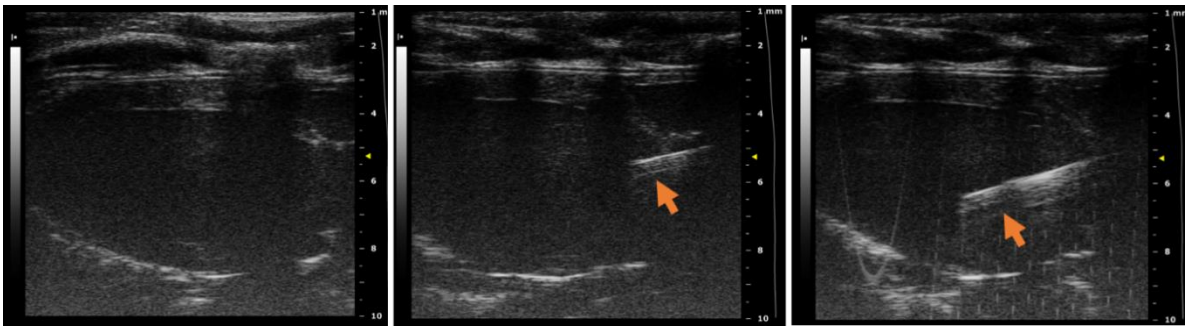

Figure 10. Mouse aortic valve wire injury procedure. Representative B-mode echocardiographic images. (Left) Left ventricle with no wire insertion. (Middle) Wire at the aortic valve level. (Right) Wire inserted in the left ventricle. Arrow indicates wire.

In the current thesis Apoe$^{-/-}$ mice fed a chow diet as well as the aortic valve wire injury model were used to put the results in both a hyperlipidemic and a non- hyperlipidemic context.

3.2.3 Mouse echocardiography

High-resolution echocardiography is currently the pillar technique for *in vivo* evaluation and assessment of aortic valve function in mice[222]. The fact that this method is a non-injurious and non-invasive technique, combined with the low degree of sedation needed, facilitates its use for longitudinal studies where multiple measurements are needed in each individual animal.

Mouse echocardiography makes it possible to obtain information on disease severity, valve morphology, leaflet calcification and valve functional performance. By using Doppler measurements, the transaortic peak velocity and the transvalvular pressure gradients can be assessed. Moreover, the B-mode (or 2D-mode) and M-mode (or 1D-mode) allows to study cusp separation at each heartbeat as well as changes in the left ventricular structure and function. However, the evaluation of changes in cusp separation requires an extensive training of the investigator and a large sample size in order to achieve reliable results and statistical power between groups[209].

3.2.4 Mouse aortic valve morphological analysis

As mentioned before, two of the hallmarks of AVS are the increased aortic valve leaflet thickness and increased calcification. In order analyze those parameters in mice, the aortic valve is sectioned from the first millimeter of the aortic root. Aortic valve leaflet thickness is studied as the average of the maximal thickness of each available cusps in all collected levels as previously described[223]. Similarly, the average of the area of each available leaflet is calculated at each collected level.

Leaflet calcification is measured by Alizarin red staining. This method is further discussed in the following sections of this chapter.

3.3 INTIMAL HYPERPLASIA – *IN VIVO* MODELS

Two mouse models of intimal hyperplasia have been described in order to investigate the pathophysiological features of intimal hyperplasia. The one used in the current thesis, is generated by a permanent carotid ligation. The other, by endothelial denudation through mechanical injury to the vessel wall.

The carotid ligation model is mostly performed in the left carotid artery by a complete ligation in the proximity of the bifurcation[224, 225]. This induces a complete obstruction of the blood flow and the formation of a thrombus which activates the endothelial layer. The neointima layer, which develops as a consequence of the ligation after 2 to 4 weeks post-surgery, is characterized, inflammatory cell infiltration and VSMC proliferation leading to complete occlusion of the vessel lumen distal to the ligation. One of the advantages of this model is the high reproducibility due to the lack of complex surgical manipulations. However, this model does not mimic in its totality the complications observed in the clinic after for example CABG failure. As an alternative, a partial ligation of the carotid artery can be performed thus avoiding the complete occlusion of the vessel lumen in order to resemble to a larger extent the clinical complication[226].

The endothelial denudation method for intimal hyperplasia is performed by passing a spring wire through the carotid or femoral artery three times[227, 228]. The damage to the endothelial layer induces the formation of a monolayer of platelets at the site of injury followed by a subsequent activation of the VSMCs, which leads to the formation of a neointima layer within 2 weeks after injury. This method resembles in a higher extent the observed complications in a clinical set, however, high technical expertise is required as well as a large sample size in order to avoid high variability and perform consistent measurements.

Other methods to induce intimal hyperplasia, for example by a balloon injury, are done in other species such as rats[229] or rabbits[229]. However, due to the size of the available balloons, those techniques are not an alternative in small rodents.

3.4 ATHEROSCLEROSIS – *IN VIVO* MODELS

Since wild-type (WT) mice are resistant to develop atherosclerosis, genetically modified hyperlipidemic mouse models are used for the study of this disease. The most common and widely used models are the Ldlr$^{-/-}$ [230] and the Apoe$^{-/-}$ mice[231, 232], the latter used in this thesis.

LDLr is primarily expressed in hepatocytes and functions by binding to the ApoE and ApoB-100 in the circulating LDL and intermediate density lipoprotein (IDL), thus promoting its immediate clearance from the circulation. In Ldlr$^{-/-}$ as well as in Apoe$^{-/-}$ mice, the disruption of the cholesterol metabolism induces an increased and sustained level of circulating cholesterol which initiates the atherosclerotic plaque formation[233]. Interestingly, Ldlr$^{-/-}$ mice develop atherosclerotic plaques only when fed a high fat diet, however, Apoe$^{-/-}$ mice develop atherosclerosis without the need of high fat diet supplementation.

Despite the differences in lipid profile between Apoe$^{-/-}$ mice and atherosclerosis patients, it is important to mention that the atherosclerotic plaques in Apoe$^{-/-}$ mice resemble in a high extent to human atherosclerotic plaques by means of having lipid accumulation, inflammatory cell infiltration, a fibrous cap rich in VSMC, necrotic cores and importantly for this thesis, plaque calcification in mature stages[234].

3.5 CALCIFICATION AND CELL PROLIFERATION

3.5.1 Calcification

In vitro models are used in order to elucidate the biological actions and molecular mechanisms of pharmacological interventions in the context of vascular and valvular calcification and cell proliferation. In the experiments presented in this thesis, VIC calcification was induced by a high Pi supplementation (2.6 mM) during 9 days[202]. Pi binds with the calcium present in the medium and precipitates into calcium phosphates. However, this precipitation is actively inhibited by the action of the calcification inhibitors secreted by the VICs. Nevertheless, when Pi is increased by external supplementation precipitation of calcium-phosphate crystals will occur, thus activating VIC osteogenic programs promoting the differentiation into oVICs. This can be observed by the increased expression of BMP2 and Runx2[235].

3.5.2 Proliferation

In this thesis the WST-1 reagent was used in order to study cell proliferation. WST-1 is a stable tetrazolium salt which is cleaved to a soluble formazan molecule (dye) by the action of succinate-tetrazolium reductase, an enzyme that is part of the respiratory chain of the mitochondria, and only functions in metabolic active cells. The formazan molecule product of the reaction has a purple color which can be quantified by spectroscopy and correlated with the amount of alive cells.

3.6 QUANTIFICATION OF CALCIFICATION AND LIPID DEPOSITION

3.6.1 Calcification

In order to detect and quantify calcification in cardiovascular diseases several methods are available. The Alizarin red staining is based on an anthroquinone dye that binds to the present calcium ions in the sample, providing an intense red color. This method can be used to assess calcification both in histological sections as well as in *in vitro* systems. Another calcification staining that is widely used is the Von Kossa staining, in which silver ions bind to phosphate in an acidic environment and provide a characteristic black color. Despite being commonly used for assessing calcification in for example atherosclerotic plaques[236], Von Kossa staining should be used with care when studying calcification in tissues from mice on a C57/BL6 background, due to the local presence of black melanocytes within the for example the aortic valve leaflets that can give rise to erroneous measurements. For this reason, in the experiments presented in this thesis only Alizarin red staining was used for assessing calcification in histological sections, both in aortic valves and atherosclerotic plaques.

To study *in vitro* calcification, other methods apart from the previously described can be used. For example, using the Osteoimage mineralization assay, method that specifically detects hydroxyapatite crystals with a fluorescent label, which can be quantified by fluorescence intensity detection.

3.6.2 Lipid deposition

Due to the nature of the atherosclerotic plaque composition, lipid deposition is commonly measured to assess plaque size. This is studied by staining histological sections of the proximal part of the aortic root with Oil Red O (ORO). ORO stains lipids with a bright red color. This method can be used either in tissue sections as well as in *in vitro* systems.

3.7 FATTY ACIDS AND LIPID MEDIATORS ANALYSIS

Several methodologies have been used in the experiments presented in this thesis in order to characterize the presence of different PUFA as well as lipid mediators in aortic valves and atherosclerotic plaques. Gas chromatography was performed for fatty acid analysis, specifically to explore the presence of n-6 and n-3 PUFA in human aortic valves as well as mouse myocardial tissue. Moreover, time-of-flight secondary ion mass spectrometry (TOF-SIMS), a method that provides molecular information of solid sample surfaces at spatial resolutions down to the sub-micrometer regime[237], was used for fatty acid profiling in mouse aortic valves, mouse atherosclerotic plaques as well as mouse myocardium. Moreover, since LC-MS/MS allows for the separation, identification and quantification of n-6 and n-3 PUFA-derived lipid mediators, this methodology was used for lipid mediator identification and quantification in human aortic valves.

4 RESULTS AND DISCUSSION

Understanding the molecular and biological mechanisms behind how SPMs mediate their actions in cardiovascular diseases may further advance our understanding on how to stimulate the resolution of inflammation. This thesis unraveled some of the effects of n-3 PUFA and the actions of the EPA-derived SPM RvE1 signaling through ChemR23 in AVS (**Article I**), intimal hyperplasia (**Article II**) and atherosclerotic plaque calcification (**Article III**).

n-3 PUFA, RvE1 and other SPMs are dysregulated in human cardiovascular diseases

Starting in the context of AVS, we observed by gas chromatography that calcified regions of human aortic valves from AVS patients contained significantly lower amounts of n-3 PUFA compared with non-calcified regions. In addition, patients with fast AVS progression exhibited a trend towards lower incorporation of n-3 PUFA in non-calcified regions compared with patients with a slow progression.

Since n-3 PUFA serve as precursor molecules for SPM biosynthesis, we also studied the presence of several SPMs in human aortic valves by LC-MS/MS. In our hands, and importantly for the first time, RvE1 and RvD3 were locally detected in aortic valve tissue. Moreover, RvE1 was significantly decreased in calcified regions compared with non-calcified regions of the aortic valve. RvD3 was also lower in calcified regions. However, the difference did not reach statistical significance.

Consistent with previous studies[64, 238], the AA-derived LTB_4 was found to be increased in calcified regions compared with non-calcified regions. LTB_4 is a potent chemoattractant derived from the 5-lipoxygenase (5-LO) metabolism of AA, demonstrated to be detrimental not just in AVS, but also in intimal hyperplasia[239] and atherosclerosis[240], diseases that are also characterized by the presence of inflammatory cells.

Overall, these results point to a non-resolved local inflammation in calcified regions of aortic valves, marked by a decreased RvE1/LTB_4 ratio, which may be caused either by the impaired incorporation of n-3 PUFA in the aortic valve tissue or by a dysfunctional lipoxygenase metabolism into the downstream SPMs. Indeed, the ratio of resolvins and leukotrienes has previously been proposed as a marker of cardiovascular risk in several studies. For example, the RvD1/LTB_4 ratio has been found to be lower in human vulnerable atherosclerotic plaques and the restoration of the ratio protects against plaque progression[172]. In another study, patients with a high salivary RvD1/LTB_4 ratio have been shown to have a significantly lower intima

media thickness compared to patients with a low ratio, suggesting that RvD1/LTB_4 can serve as a marker of non-resolved inflammation in the context of sub-clinical atherosclerosis[241]

Transcriptomic analysis of aortic valves revealed that ChemR23 was the highest expressed receptor in the aortic valve compared with the RvD1 and RvD3 receptors ALX/FPR2, as well as GPR32, and with the RvD2 receptor GPR18. ChemR23 protein expression was further studied by immunohistochemistry (IHC) and by immunofluorescence (IF) confocal microscopy, showing co-localization with the VIC markers α-SMA as well as vimentin (Figure 11). It is important to mention that ChemR23 in atherosclerotic plaques co-localizes with the VSMC marker α-SMA in a similar manner[199].

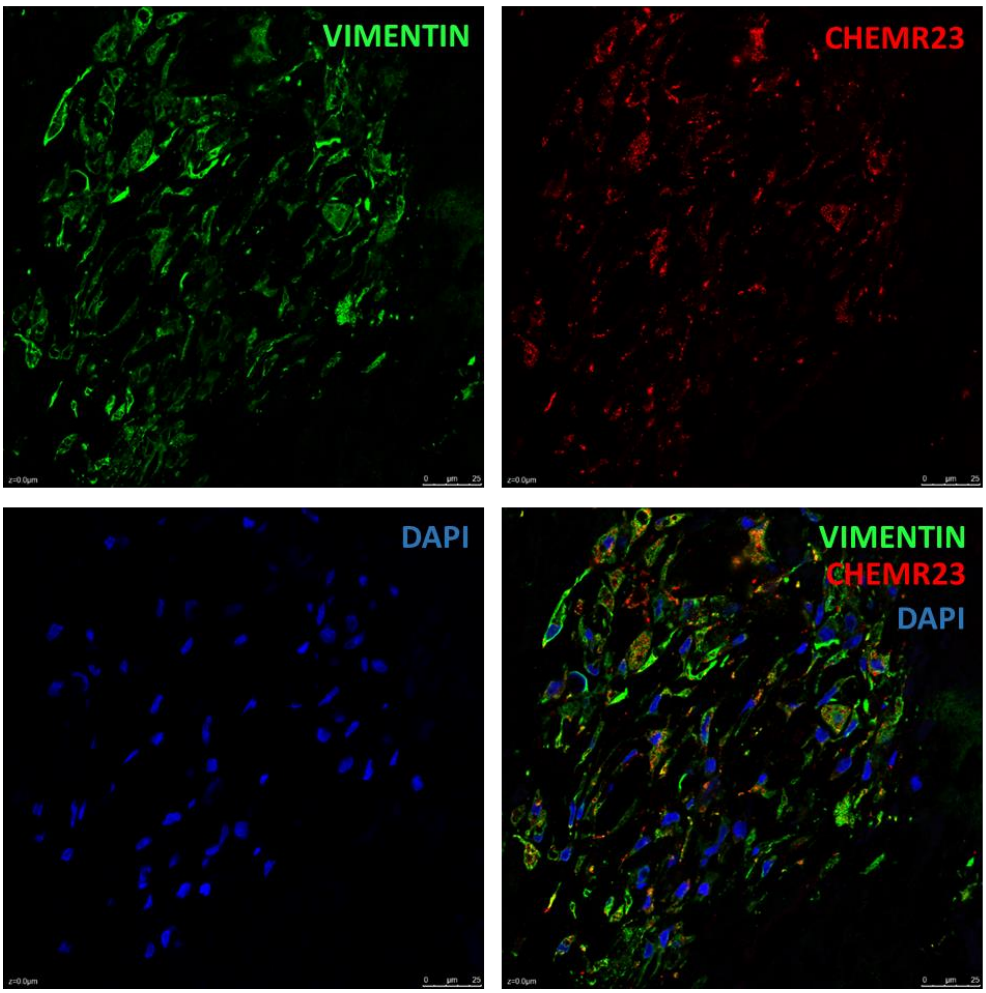

Figure 11. ChemR23 is expressed in human aortic valves. Representative immunofluorescence micrographs of human aortic valves. Vimentin (green), ChemR23 (red), and co-localization. Nuclei are stained blue.

One important hallmark of AVS and atherosclerosis is calcification. Knowing that RvE1 is dysregulated in calcified regions of aortic valves together with the observation of ChemR23 being highly expressed in VICs, we aimed to unravel the potential role of RvE1 in decreasing VIC calcification. Interestingly RvE1 treatment significantly decreased VIC calcification

induced by a high Pi concentration *in vitro* (Figure 12). This effect could be partially explained by extrapolating previous findings in VSMCs, where RvE1 also reduces calcification via downregulating BMP2 expression, hallmark of osteoblastic differentiation of vascular cells[202].

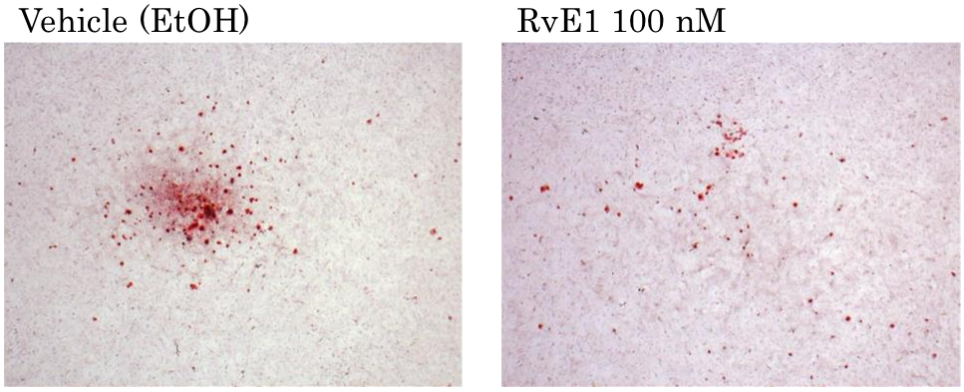

Figure 12. RvE1 decreases VIC Pi-induced calcification after 9 days of treatment.

n-3 PUFA reduce AVS, atherosclerotic plaque calcification and intimal hyperplasia in mice

Next, *in vivo* experiments were performed in order to decipher the mechanisms behind the n-3 PUFA/ RvE1/ChemR23 axis in cardiovascular disease. We first inserted the Fat-1 transgene (Fat-1^{tg}), encoding a n-3 PUFA desaturase which allows the conversion of dietary n-6 PUFA into n-3 PUFA, in the $Apoe^{-/-}$ mouse model of atherosclerosis and AVS. We also inserted the Fat-1^{tg} in a normolipidemic background mouse ($Apoe^{+/+}$) in order to elucidate the effects of n-3 PUFA in a mouse model for intimal hyperplasia independent of hypercholesterolemia.

We and other research groups have previously described that exogenous supplementation of n-3 PUFA increases RvE1 formation[199], as well as endogenous supplementation due to the presence of Fat-1^{tg} [242, 243]. However, the effects of RvE1 have not been addressed previously in the context of AVS nor atherosclerotic plaque calcification.

TOF-SIMS analysis revealed that n-3 PUFA incorporated in the aortic valve and atherosclerotic lesions of Fat-1^{tg}x$Apoe^{-/-}$ mice. It is important to note that no evidence showing n-3 PUFA incorporation in the aortic valve of Fat-1^{tg} mice has previously been reported in the literature. As expected, the increase in n-3 PUFA was accompanied by a decrease in n-6 PUFA, observations that were further explored and confirmed in myocardial tissue of the same mice by gas chromatography. Interestingly, EPA and AA in Fat-1^{tg} mouse atherosclerotic plaques were the only PUFA that remained significantly statistically different compared with $Apoe^{-/-}$ mice after multiple test adjustment, suggesting a potential dominant role of EPA in the

atherosclerotic plaque. Indeed, these results are in line with the previously mentioned clinical trial showing that EPA supplementation in high doses significantly reduces cardiovascular risk[131] and induces atherosclerotic plaque regression[178]. Moreover, TOF-SIMS fatty acid profiling of myocardial tissue mirrored the atherosclerotic plaque profile demonstrating that the fatty acid signature in the myocardium reflects the content in the atherosclerotic plaque.

Further characterization of atherosclerotic plaques by TOF-SIMS revealed that local cholesterol in atherosclerotic plaques is mostly present in the form of free cholesterol and not as cholesterol esters, a form that was found in the blood remnants surrounding the atherosclerotic plaques. Moreover, no differences between total cholesterol amounts were observed between Apoe$^{-/-}$ mice carrying and not carrying the Fat-1^{tg}.

Knowing that Fat-1^{tg} mice incorporated n-3 PUFA within the aortic valve, we next characterized the AVS development in Apoe$^{-/-}$ mice with and without the Fat-1^{tg} by echocardiography from 52 until 72 weeks of age. Fat-1^{tg}xApoe$^{-/-}$ mice exhibited reduced AVS progression, having reduced transaortic peak velocity and increased cusp separation. Moreover, aortic valve histological analysis showed that Fat-1^{tg}xApoe$^{-/-}$ mice exhibited a reduced cross-sectional aortic valve leaflet area as well as calcification (calcification depicted in Figure 13A) compared with non-transgenic Apoe$^{-/-}$ mice at 72 weeks. Despite not showing reduced atherosclerotic plaque lesion size, probably due to the advanced age of the mice, Fat-1^{tg}xApoe$^{-/-}$ mice also exhibited reduced atherosclerotic plaque calcification compared with Apoe$^{-/-}$ mice (Figure 13B). These results are in line with previous observations from other groups showing that exogenous EPA supplementation decreases arterial calcification in Klotho$^{-/-}$ mice[207].

Furthermore, by using a model of a permanent left carotid ligation in normolipidemic mice, we demonstrated that Fat-1^{tg} also induced beneficial effects by reducing intimal hyperplasia formation after 4 weeks of ligation compared with WT (ChemR23$^{+/+}$) mice (Figure 13C). This observation is of particular importance since the reduction of intimal hyperplasia induced by Fat-1^{tg} was not dependent on the lipid lowering effects of n-3 PUFA.

ChemR23 deletion increases AVS, atherosclerotic plaque calcification and intimal hyperplasia

In order to study whether the beneficial effects induced by Fat-1^{tg} in AVS, atherosclerotic plaque calcification and intimal hyperplasia were mediated by the n-3 PUFA downstream SPM RvE1 signaling through ChemR23, we generated the ChemR23-deficient mouse (ChemR23$^{-/-}$) in a Apoe$^{-/-}$ and normolipidemic background. ChemR23$^{-/-}$ mice were also generated in the presence of the Fat-1^{tg}.

Interestingly, $Apoe^{-/-}$xChemR23$^{-/-}$ mice exhibited a more pronounced AVS development compared with $Apoe^{-/-}$ mice at 72 weeks, observed by the significant increase in transaortic peak velocity, reduced cusp separation as well as increased cross-sectional aortic valve leaflet area and calcification (calcification depicted in Figure 13A) in histological examinations. The detrimental effects of ChemR23 deletion in AVS were further explored in a normolipidemic model ($Apoe^{+/+}$) of AVS induced by a direct aortic valve wire injury. Consistent with the previous results, after 16 weeks of valve injury ChemR23$^{-/-}$ mice exhibited a higher transaortic peak velocity as well as increased leaflet thickness compared with ChemR23$^{+/+}$ mice.

In similarity to the observations in valves, $Apoe^{-/-}$xChemR23$^{-/-}$ mice significantly increased atherosclerotic plaque size and calcification compared with $Apoe^{-/-}$ mice (Figure 13B). This is not the first observation demonstrating the beneficial role of ChemR23 in the development of atherosclerosis. Indeed, our laboratory has previously demonstrated the protective role of ChemR23 as an antiatherogenic receptor in macrophage signaling by means of decreasing inflammation, reducing oxLDL uptake, and enhancing phagocytosis. These effects translate into the observed reduced mouse atherosclerotic plaque size, as well as a reduced macrophage content and necrotic core formation[199].

In the same line, in the context of intimal hyperplasia, ChemR23$^{-/-}$ mice developed larger lesions compared with WT (ChemR23$^{+/+}$) mice 4 weeks after a carotid ligation (Figure 13C).

RvE1 reduces AVS and atherosclerotic plaque calcification through ChemR23

Aortic valve n-3 PUFA incorporation by the presence of Fat-1^{tg} in $Apoe^{-/-}$xChemR23$^{-/-}$ mice did not improve echocardiographic parameters compared with $Apoe^{-/-}$xChemR23$^{-/-}$ and did not reduce cross-sectional aortic valve leaflet area nor calcification (calcification depicted in Figure 13A).

Interestingly, all-groups adjusted correlations between quantitative echocardiographic and histological parameters (cross-sectional aortic valve leaflet area and leaflet calcification) were statistically correlated, as well as the adjusted correlation between the histological parameters cross-sectional aortic valve leaflet area and leaflet calcification. However, partial correlations (which measure the level of association between two variables whilst removing the effect of third variables) between quantitative variables (for example, cross-sectional aortic valve leaflet area and leaflet calcification) showed non-significant correlations when excluding genotype effect. The lack of significance of the partial correlations between the different quantitative measures, indicates that the beneficial effects observed by the presence of Fat-1^{tg} as well as the detrimental effects induced by ChemR23 deletion may only be driven by the genotype effect,

which suggests that the distinct observations between the different genotypes were caused through independent mechanisms.

In a similar manner, atherosclerotic plaque calcification in Fat-1^{tg}xApoe$^{-/-}$xChemR23$^{-/-}$ mice was not reduced compared with Apoe$^{-/-}$xChemR23$^{-/-}$ mice (Figure 13B).

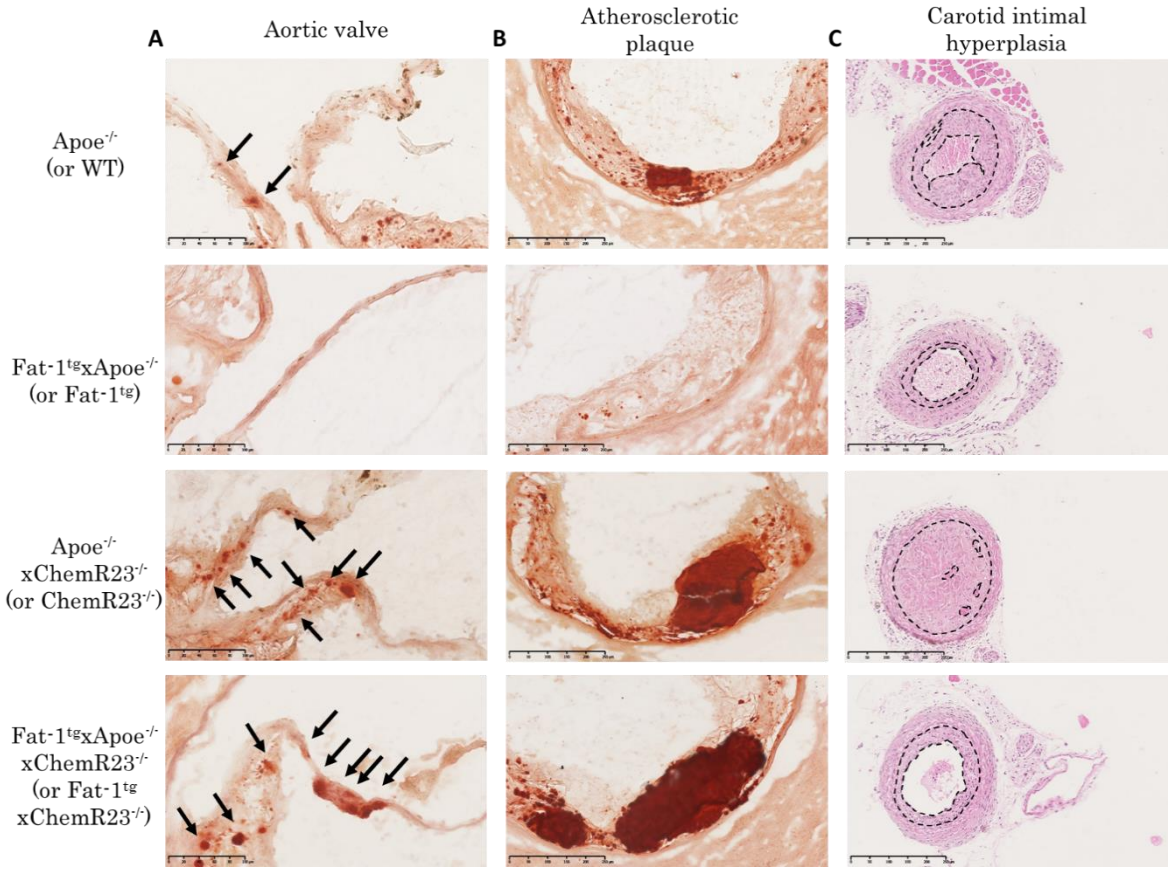

Figure 13. Fat-1^{tg} reduces aortic valve and atherosclerotic plaque calcification as well as intimal hyperplasia, whereas targeted deletion of ChemR23 increases aortic valve and atherosclerotic plaque calcification as well as intimal hyperplasia in mice. (A) Aortic valve leaflets and (B) atherosclerotic plaque calcification stained with Alizarin red of 72 weeks old Apoe$^{-/-}$, Fat-1^{tg}xApoe$^{-/-}$, Apoe$^{-/-}$xChemR23$^{-/-}$ and Fat-1^{tg}xApoe$^{-/-}$xChemR23$^{-/-}$ mice. (C) Carotid intimal hyperplasia in 14 weeks old WT, Fat-1^{tg}, ChemR23$^{-/-}$ and Fat-1^{tg}xChemR23$^{-/-}$ mice, 4 weeks after carotid artery ligation.

Overall, our results show that the beneficial effects of Fat-1^{tg} in AVS and atherosclerotic plaque calcification were only observed in the presence of ChemR23, suggesting that the effects in both conditions were mediated by RvE1 signaling through its receptor. As a matter of fact, the important role of RvE1in atherosclerosis development has been previously explored in a study that shows that RvE1 treatment reduces atherosclerotic lesion size in hyperlipidemic mice at younger ages[244].

n-3 PUFA reduce intimal hyperplasia but not exclusively through ChemR23

In contrast with the observations in AVS and atherosclerotic plaque calcification, Fat-1^{tg} also reduced intimal hyperplasia in ChemR23$^{-/-}$ mice, suggesting that n-3 PUFA do not reduce intimal hyperplasia development solely by signaling via RvE1 through ChemR23 (Figure 13C). Indeed, the effects of different SPMs in the development of intimal hyperplasia may be receptor-specific in both inflammatory and structural cells. A recent study has shown that RvE1 treatment in mice protects against intimal hyperplasia after femoral artery injury by signaling, not through ChemR23, but through BLT1 in leukocytes to reduce the infiltration of monocytes, neutrophils as well as T-cells to the site of injury. In contrast, RvE1 treatment reduces VSMCs migration signaling through ChemR23, and not through BLT1[201].

The fact that Fat-1^{tg} also reduced intimal hyperplasia in the absence of ChemR23 potentially suggests that the beneficial effects may come from other n-3 PUFA-derived SPMs. Other SPMs have been described as having a beneficial role in the development of intimal hyperplasia. For example, administration of RvD1 and PD1 in rats and rabbits after artery balloon injury reduces macrophage, T-cell and neutrophil infiltration[229, 245, 246]. Moreover, other preclinical studies have shown that RvD2 and Mar1 induce M2 macrophage polarization after carotid ligation[225]. Furthermore, RvD1 and RvD2 reduce VSMC migration and proliferation as well as reduce collagen deposition[246, 247] and NFκB activation in inflammatory cells, mitigating the inflammatory environment. This translates to reduced expression levels of IL-1β, IL-6[248] and MCP1[225]. It is important to note that other studies have described a direct role of n-3 PUFA in decreasing intimal hyperplasia by directly binding to the free-fatty acid receptor-4[249], which suggest another potential beneficial role of n-3 PUFA independent of SPM generation.

In summary, our results revealed a protective role of n-3 PUFA supplementation and a detrimental role of ChemR23 deletion both in AVS, atherosclerotic plaque calcification and intimal hyperplasia. Interestingly, a specific dominancy of RvE1 signaling through ChemR23 was only observed in the context of AVS and atherosclerotic plaque calcification, with a reduction of cross-sectional aortic valve leaflet area and calcification as well as atherosclerotic plaque calcification.

Macrophage ChemR23 deletion promotes inflammation

So far, the results presented in this thesis have demonstrated the detrimental effect of ChemR23 deletion in inflammatory mouse models for AVS, atherosclerotic plaque calcification and intimal hyperplasia. However, a previous study from our laboratory has shown that the presence of ChemR23 is associated with increased vascular calcification in a non-inflammatory

model of medial calcification induced by high doses of vitamin D3[202]. This effect was explained to be mediated by a direct VSMC phenotypic switching into a synthetic and proliferative phenotype, coupled to the presence of ChemR23.

This was further explored in this thesis, where it was observed that indeed, ChemR23$^{+/+}$ mouse VSMCs exhibited a significantly higher proliferation than ChemR23$^{-/-}$ VSMCs *in vitro*. However, VSMCs treated with conditioned media from ChemR23$^{-/-}$ macrophages proliferated more than VSMCs treated with media from ChemR23$^{+/+}$ macrophages. A later macrophage characterization demonstrated a more pro-inflammatory phenotype of ChemR23$^{-/-}$ compared with ChemR23$^{+/+}$ macrophages, marked by the significantly increased levels of tumor necrosis factor (TNF) α, MMP9 and a trend for IL-6. For this reason, we concluded that the increased detrimental effects mediated by ChemR23 deletion in the *in vivo* models of AVS, atherosclerosis and intimal hyperplasia may be mediated by the pro-inflammatory macrophage effects.

RvE1 signaling through ChemR23 promotes M2 macrophage polarization

Distinct macrophage subpopulations are known to play different roles in atherosclerotic plaques [250] and stenotic aortic valves[251]. We studied the effects of n-3 PUFA and ChemR23 in macrophage polarization into the M1 and M2 subtypes by IHC, both in mouse aortic valves and atherosclerotic plaques.

The characterization of macrophage populations in the mouse aortic valves revealed a higher expression of Arg1 as well as CD206 only in the presence of Fat-1^{tg}, suggesting that high levels of n-3 PUFA increased macrophage polarization into M2 phenotype. Of note, Fat-1^{tg} did not induce M2 polarization in the absence of ChemR23, once again showing that the beneficial effects of Fat-1^{tg} were only present in mice with an intact ChemR23 expression. Lending support to this finding, mRNA levels of ChemR23 in human stenotic valves, both in non-calcified and calcified tissue, were significantly correlated with the expression of several M2 macrophage markers such as CD206, heme oxygenase (HMOX), CD163, CD209 and CD200R1.

Furthermore, and in line with our observations in aortic valves, higher levels of Arg1 were found in atherosclerotic plaques of Fat-1^{tg}xApoe$^{-/-}$ mice compared with Apoe$^{-/-}$ mice, as well as compared with Apoe$^{-/-}$ mice deficient in ChemR23, independently of the presence of Fat-1^{tg}. This further reinforces the idea that macrophage polarization was mediated by RvE1 signaling through ChemR23, both in atherosclerotic plaques as well as in aortic valves.

Apart from being a marker of M2 macrophage phenotype, Arg1 has been described to play an important role in vascular calcification. As previously described, macrophages activated by high concentrations of Pi acquire anti-calcifying properties accompanied by a sharp increase in Arg1 and, interestingly, also of ChemR23[153]. These results are of particular importance for this thesis when taking into account recent evidence showing that Arg1 promotes macrophage efferocytosis of apoptotic cells by metabolizing the engulfed cell-derived arginine into putrescine. Putrescine activates macrophage Rac1 expression and facilitates further apoptotic cell internalization by promoting actin polymerization, thus increasing the resolution of the inflammatory process[252, 253]. This suggests that Arg1 expression, which in our experiments was induced by increased levels of n-3 PUFA solely in the presence of ChemR23, could potentially be considered a functional marker of macrophages with anti-calcifying and pro-resolving actions (Figure 14).

Pi-activated macrophages mediate their anti-calcifying actions in part by reducing TNAP expression, thus promoting the accumulation of PPi in the extracellular space[154]. In line with this, we observed a trend towards a decreased mRNA expression of TNAP in the aorta of $Apoe^{-/-}$ mice with enhanced n-3 PUFA levels by the insertion of $Fat\text{-}1^{tg}$. Moreover, ChemR23 deletion significantly increased TNAP expression in aortas of $Fat\text{-}1^{tg}xApoe^{-/-}$ mice. This further supports the role of RvE1 in reducing atherosclerotic plaque and valvular calcification through ChemR23, probably by limiting the hydrolysis of PPi into Pi (Figure 14).

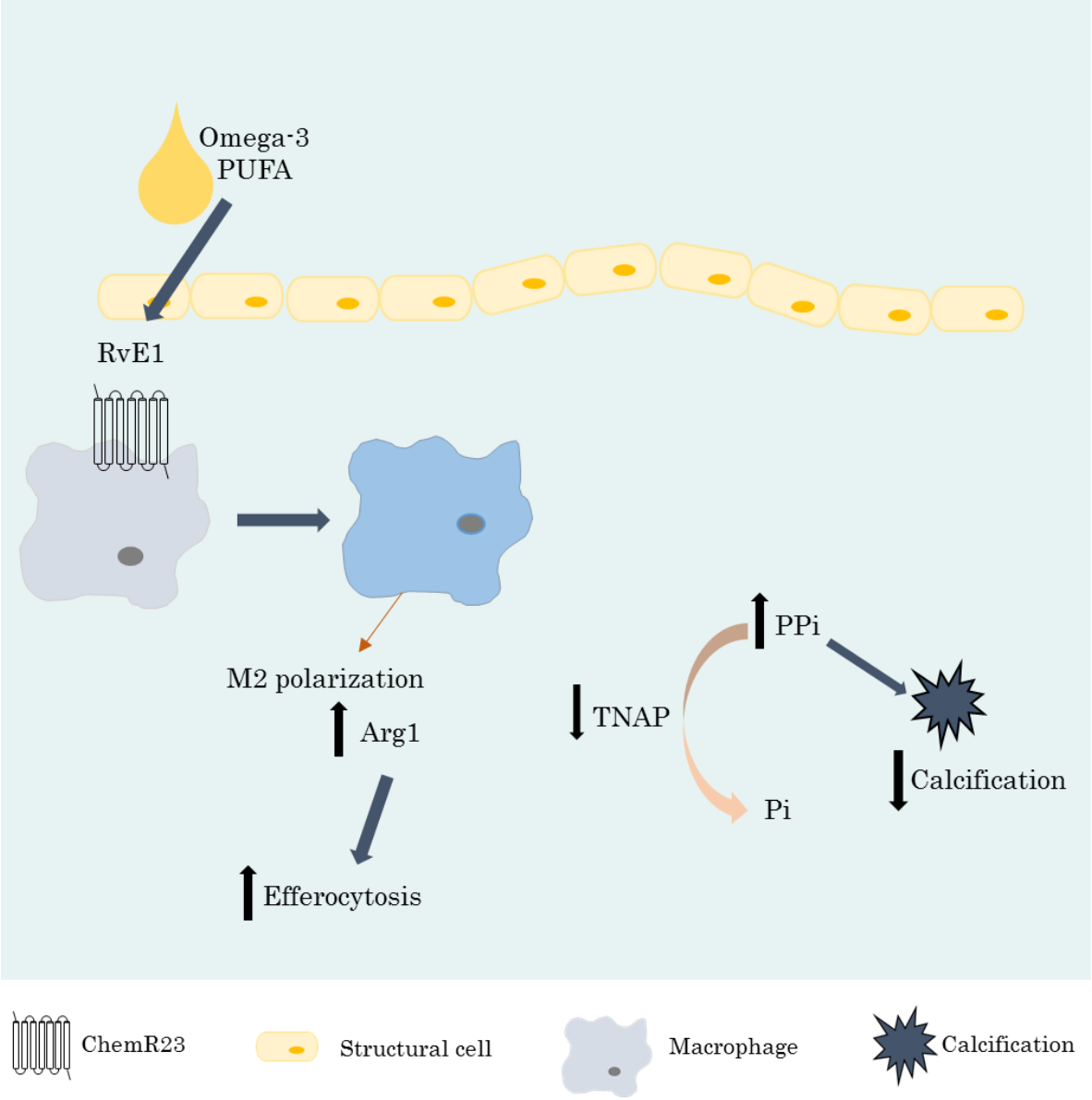

Figure 14. The n-3 PUFA-derived RvE1 signaling through ChemR23 reduces cardiovascular calcification. Effects include increased M2 macrophage polarization, marked by the increased expression of Arg1, a protein associated with increased efferocytosis, as well as reduced TNAP expression levels, favoring the accumulation of the calcification inhibitor PPi only in the presence of ChemR23.

5 CONCLUDING REMARKS

Previous experimental studies have shown that n-3 PUFA exert a large number of beneficial effects in cardiovascular inflammation. As discussed throughout this thesis, one potential underlying mechanism relies in serving as a substrate for the biosynthesis of SPMs, which have been demonstrated to exert beneficial actions in several cardiovascular complications. The applicability of n-3 PUFA as a potential therapy in cardiovascular diseases has been supported in recent clinical trials showing a decreased risk of cardiovascular events, including cardiovascular death, and an enhanced atherosclerotic plaque regression by high-dose supplementation of EPA[131, 178].

Throughout this thesis, the role of n-3 PUFA, and specifically, the downstream EPA-derived SPM RvE1 signaling through its receptor ChemR23 has been studied in the context of AVS, intimal hyperplasia and atherosclerotic plaque calcification.

In **Article I**, we provide evidence for the first time that n-3 PUFA accumulates both in human and murine aortic valves. Moreover, we describe that n-3 PUFA and RvE1 are decreased in calcified regions of human valves. In mice, increasing n-3 PUFA levels by the presence of Fat-1^{tg} halt AVS progression, reduce cross-sectional aortic valve leaflet area and calcification as well as promote macrophage polarization into the anti-inflammatory M2 phenotype, and importantly, only in the presence of ChemR23. Furthermore, ChemR23 deletion enhances AVS progression, and increases cross-sectional aortic valve leaflet area and calcification, effects that were not regressed by increasing the levels of n-3 PUFA, suggesting that the beneficial actions were mediated by the activation of the RvE1/ChemR23 signaling axis.

In **Article II**, we established that deletion of ChemR23 increases intimal hyperplasia in mice. This effect may be caused by the fact that ChemR23$^{-/-}$ macrophages exhibit a more pro-inflammatory phenotype, characterized by the increased expression of pro-inflammatory cytokines such as TNFα as well as MMP9.

Interestingly, n-3 PUFA supplementation by the presence of Fat-1^{tg} reduces intimal hyperplasia. However, n-3 PUFA beneficial effects were also observed in mice lacking the RvE1 receptor ChemR23, suggesting that other SPMs as well as other receptors apart from RvE1 and ChemR23, respectively, may have potential beneficial roles in this context.

In **Article III**, we show that n-3 PUFA in mice are actively incorporated in the atherosclerotic plaque. We also decipher that a correct signaling through ChemR23 plays a critical role in the development of atherosclerotic plaque calcification. As a matter of fact, increased n-3 PUFA

reduces atherosclerotic plaque calcification, and the deletion of ChemR23 increases calcification, independently of the presence of Fat-1^{tg}. The beneficial effects of n-3 PUFA may be mediated by a macrophage polarization into the anti-inflammatory/anti-calcification M2 phenotype, marked by an increased expression of Arg1. Moreover, ChemR23 deletion significantly increased TNAP expression in Fat-1^{tg} mice, suggesting that the n-3 PUFA effects may be mediated by an enhanced RvE1/ChemR23 signaling.

In summary, the results presented in this thesis show the importance of the n-3 PUFA/RvE1/ChemR23 axis in cardiovascular inflammation. This axis emerges as a potential signaling pathway to be clinically evaluated in order to improve the prognosis of patients with cardiovascular diseases.

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"The mental imagery involved with pianistic tactilia is not related to the striking of individual keys but rather to the rites of passage between notes."

Glenn Gould

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